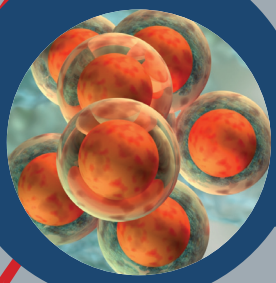

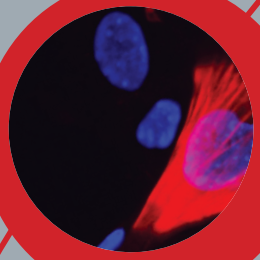

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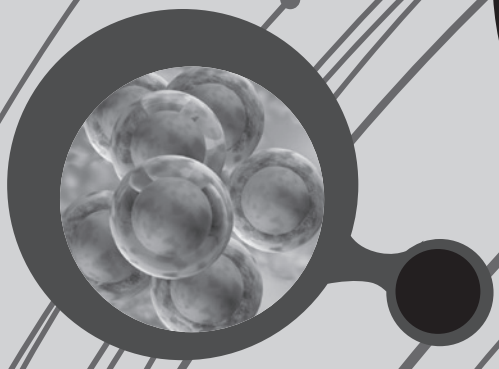

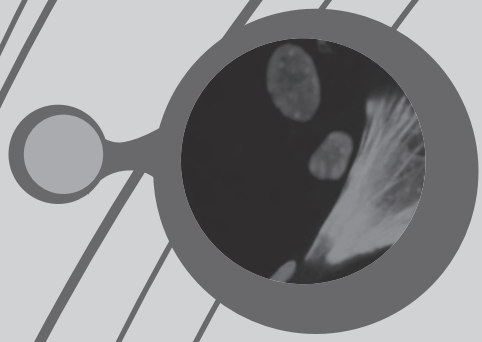

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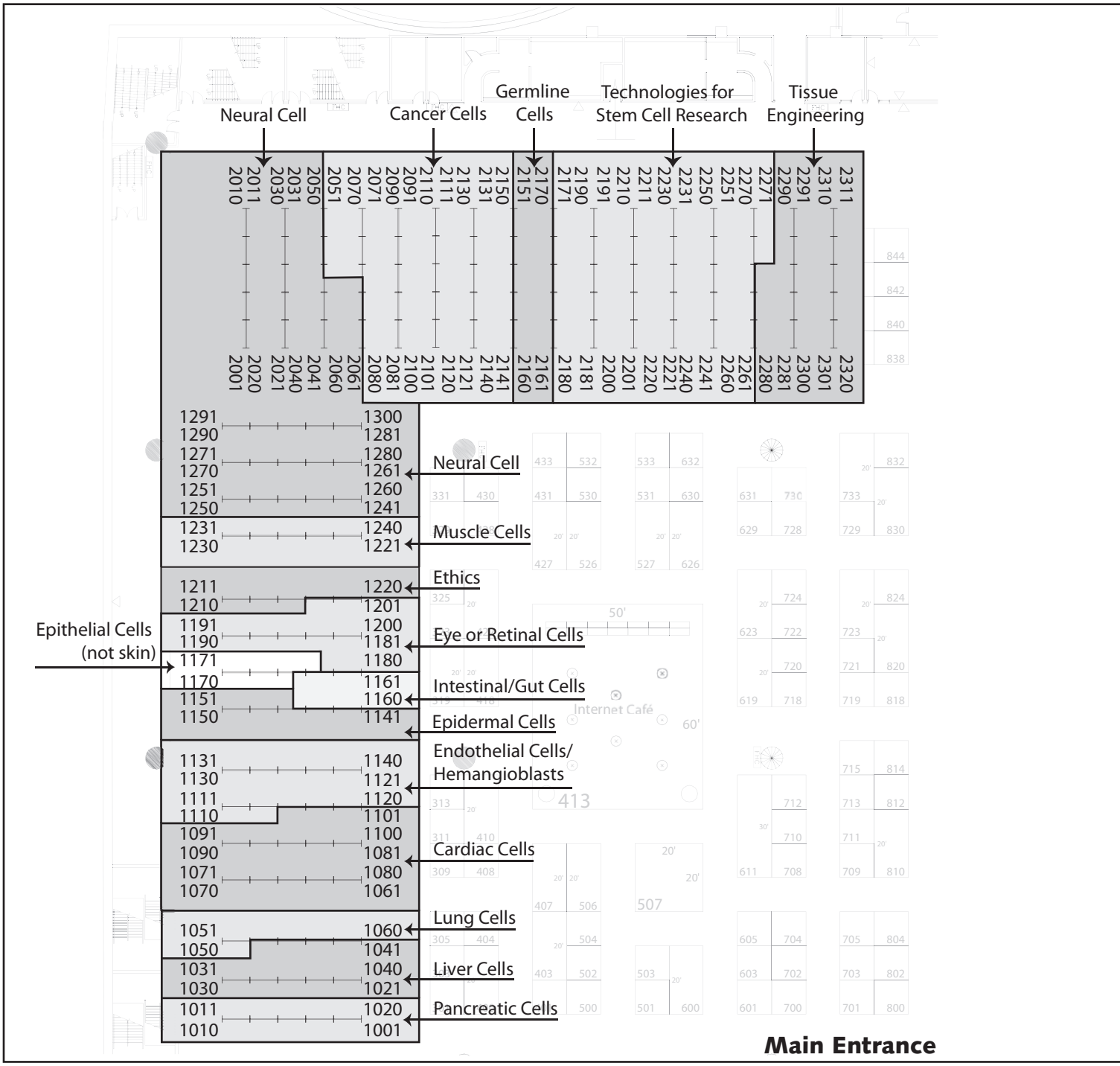
Poster Floor Plan



Poster Session Schedule

Wednesday, June 15

3:30 – 4:00 p.m.
All Posters Put On Display

4:00 – 8:00 p.m.
Posters Open for Viewing

Thursday, June 16

11:00 a.m. – 8:00 p.m.
Posters Open for Viewing

6:00 – 8:00 p.m.
Poster Presentation Reception
ALL ODD NUMBERED
POSTERS PRESENTED

Friday, June 17

11:00 a.m. – 8:00 p.m.
Posters Open for Viewing

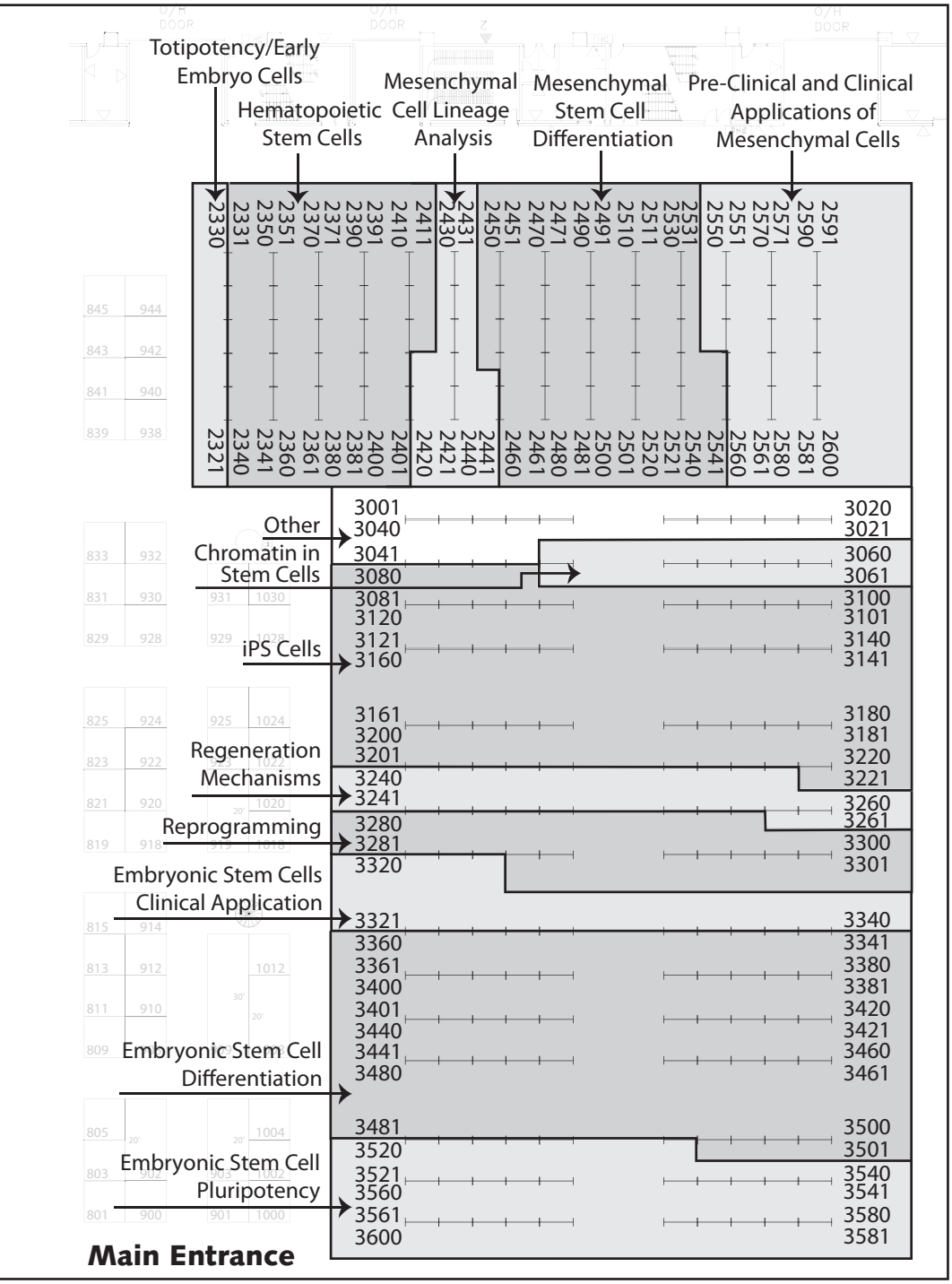
6:00 – 8:00 p.m.
Poster Presentation Reception
ALL EVEN NUMBERED
POSTERS PRESENTED

Saturday, June 18

11:00 a.m. – 4:00 p.m.
Posters Open for Viewing

4:00 p.m.
Posters Dismantle

Poster Floor Plan



Poster Boards by Topic

Posters 1001 – 1300

Posters 2001 – 2600

Posters 3001 – 3600

NO PHOTOGRAPHY OR RECORDINGS PERMITTED IN THE POSTER AREAS

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PANCREATIC CELLS

Poster Board Number: 1002

IN VIVO MATURATION OF HUMAN EMBRYONIC STEM CELL-DERIVED PANCREATIC PRECURSOR CELLS TO FUNCTIONAL ISLET-LIKE CELLS CAPABLE OF TREATING STZ-INDUCED DIABETES

Bruin, Jennifer E.[1], Riedel, Michael J.[1], Mojibian, Majid[1], Xu, Jean[2], Gauvin, Rebecca[2], Narayan, Kavitha[2], Karanu, Francis[2], Rezania, Alireza[2], Kieffer, Timothy J.[1]

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We have developed a protocol to differentiate human embryonic stem cells (hESCs) *in vitro* into pancreatic precursor cells that can be further differentiated *in vivo* towards mature pancreatic cell types following transplantation. Following a 4-stage, 14-day differentiation, hESC-derived cells were transplanted into the kidney capsule of streptozotocin (STZ)-treated diabetic SCID-Beige mice. Euglycemia was maintained in diabetic animals with subcutaneous, slow-release insulin pellets for the first 12 - 16 weeks post-transplant. After this time STZ-treated mice remained normoglycemic without an insulin pellet replacement, likely due to graft-derived C-peptide. Following transplantation, fed human C-peptide levels in diabetic mice steadily increased from 0.077 ± 0.01 to 1.99 ± 0.3 ng/mL between 4 and 30 weeks. Furthermore, engrafted cells were capable of secreting human C-peptide in response to both meal and glucose challenges by 30 weeks post-transplant. An arginine challenge stimulated significant secretion of glucagon and GLP-1, in addition to mild release of insulin. Maturation of hESC-derived pancreatic precursor cells was characterized by immunocytochemistry prior to and at several timepoints following transplantation. Prior to transplant, hESC-derived cell clusters contained a mixture of scattered cells expressing insulin alone, glucagon alone, or both hormones. At 4 weeks post-transplant, engrafted cells maintained a similar hormone expression pattern to the pre-transplant clusters, but in distinct islet-like endocrine clusters. By 12 weeks, the majority of endocrine cells in kidney grafts expressed either insulin or glucagon (but not both hormones), and displayed an architecture resembling mature adult human pancreatic islets. Furthermore, insulin/Pdx1 co-positive cells were observed near duct-like structures immunopositive for CK19 (a ductal marker), suggesting the possibility of ongoing islet neogenesis. In the islet-like endocrine clusters, insulin-expressing and glucagon-expressing cells exhibited transcription factor profiles similar to endogenous human beta and alpha cells, respectively. Taken together, these data demonstrate that hESCs can differentiate *in vivo* into a mixed population of mature pancreatic endocrine cells, capable of maintaining normoglycemia in STZ-treated mice without tumour formation.

Poster Board Number: 1004

SMALL MOLECULES INDUCE EFFICIENT DIFFERENTIATION INTO INSULIN-PRODUCING CELLS FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

Kunisada, Yuya, Tsubooka-Yamazoe, Noriko, Shoji, Masanobu, Hosoya, Masaki

Frontier Research Laboratories, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Tsukuba, Ibaraki, Japan

Human induced pluripotent stem (hiPS) cells have potential uses for drug discovery and cell therapy, including generation of pancreatic β-cells for diabetes research and treatment. There have already been several reports on the generation of insulin-producing cells from hiPS cells or hES cells *in vitro*, but there are still many challenges to overcome, including the low and variable differentiation efficiency of the current protocols for different hiPS or hES cell lines. To realize the full potential of hiPS or hES cells in the field of diabetes, it is necessary to be able to generate insulin-producing cells in an efficient and reproducible manner. Here, we report a novel protocol for generating insulin-producing cells from hiPS cells. We screened for small molecules that induced pancreatic differentiation from definitive endoderm and found that combined treatment with the compounds could induce efficient differentiation of pancreatic progenitor cells. Expression of the pancreatic progenitor markers Pdx1 and Ngn3 was increased at this step and Pdx1-positive cells were emerged. Next, we screened for small molecules that could induce insulin-producing cells from Pdx1-positive cells. Several compounds were found to induce the differentiation of insulin-producing cells. By combined treatment with these compounds, a larger number of the cells became insulin positive. With this protocol, all five hiPS cell lines that we examined showed differentiation into insulin-producing cells at a similar level of efficiency. They were also positive for antibodies to various islet-enriched transcription factors, including Pdx1, NeuroD1, Islet-1, PAX6, and Nkx2.2. Other pancreatic endocrine cells (expressing glucagon, somatostatin, and ghrelin) were also generated under the same conditions. Moreover, the differentiated cells could secrete human c-peptide in response to potassium chloride, tolbutamide (a KATP channel blocker), (-)-Bay K8644 (a L-type Ca2+ channel agonist), carbachol (a muscarinic agonist), and IBMX (a phosphodiesterase inhibitor). This differentiation protocol may contribute to drug discovery and cell therapy for diabetes.

Poster Board Number: 1006

IMPROVEMENT IN PANCREATIC ISLET FUNCTION AFTER CO-CULTURE WITH ADULT HUMAN PANCREATIC MESENCHYMAL STEM CELLS IS CELL CONTACT DEPENDENT

Yeung, Telford, Seeberger, Karen, Lyon, James, Korbutt, Gregory

University of Alberta, Edmonton, AB, Canada

Background: Islet transplantation offers metabolic benefits for type 1 diabetics, but recipients progressively lose insulin independence as a result of reduced islet function and/or destruction. Consequently, activating cellular repair may be a strategy to mitigate the resulting loss and dysfunction of islets. Bone marrow mesenchymal stem cells (BM-MSCs), which have been utilized in rodent islet transplantation, are an attractive solution due to their capacity to induce repair in various tissues. The expansion of a MSC population from the human pancreas led us to believe pancreatic MSCs (pMSCs) may possess similar regenerative properties. As therapeutic doses of MSCs exceed the natural frequency of these cells, we hypothesize that an expanded population of pancreatic MSCs may be beneficial for human islet function and survival. Methods: Mesenchymal stem cells from bone marrow and pancreas were characterized for CD29, CD34, CD45, CD73, CD90 and CD105 expression. To assess the regenerative capacity of these MSCs, five hundred human islet equivalents from donor pancreata were cultured in direct contact with MSCs by aggregating the islets with 0.5, 1.0, 1.5 or 3.0 x 106 pMSCs or BM-MSCs over a 72 hr culture period. For a non-contact culture system, human islets were alginate microencapsulated to prevent contact with MSCs and then were co-cultured on a proliferating pMSC monolayer for 72 hr. Controls included islets cultured without MSCs for 72 hr. After 72 hr, all conditions were characterized for glucose responsiveness as well as cellular insulin and DNA contents. Results: Immunophenotyping for surface antigen expression confirmed that pMSCs are similar to conventional BM-MSCs (>95% CD29, CD73, CD90 and CD105 expression along with <5% CD34 and CD45 expression). After direct contact co-culture, both pMSCs and BM-MSCs improved glucose responsiveness in contrast to islets cultured alone. Moreover, these effects were observed in a dose response manner; and the most favorable effect based on stimulation index

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(SI), the ratio of insulin release at high glucose compared to low glucose, was observed with 500 islet equivalents cultured in direct contact with 1.0 x 106 pMSCs (SIco-culture = 2.73 ± 0.31 vs. SIislets alone = 1.50 ± 0.29, n=6) or 1.0 x 106 BM-MSCs (SIco-culture = 2.53 ± 0.37 vs. SIislets alone = 1.78 ± 0.14, n=2). However, in the non-contact culture model, no beneficial effect on human islet function was observed (SIco-culture = 2.45 ± 0.50 vs. SIislets alone = 2.37 ± 0.52, n=4). In all conditions, co-culture (direct and non-direct) had no adverse or beneficial effects on cellular insulin and DNA content. Conclusions: Improved glucose responsiveness from co-cultured islets is cell contact and MSC dose dependent, suggesting that this improvement is mediated by cell to cell interactions or is a function of trophic factor secretion. Preliminary data also demonstrate that pMSCs and BM-MSCs are equivalent in their regenerative capacity for islet function because they likely secrete similar trophic factors including vascular endothelial growth factor and hepatocyte growth factor. As MSC aggregates improved β-cell function from isolated human islets, MSC-islet composites are expected to improve transplant success by reducing the marginal mass of transplanted islets to reverse diabetes or by prolonging graft function.

Poster Board Number: 1008

TREATMENT OF TYPE 1 DIABETES WITH INSULIN PRODUCING CELLS DERIVED FROM HUMAN AND MOUSE EMBRYONIC STEM CELLS

Li, Mira, Whiteley, Jennifer, Chua, Shawn, Rogers, Ian

Samuel Lunenfeld Research Institute, Toronto, ON, Canada

Type 1 diabetes affects approximately 1.3 million in the US alone and is the most common metabolic disease in children. People with diabetes are required to constantly monitor their glucose and take daily injections of insulin. Even with strict compliance patients eventually develop complications including nephropathy and retinopathy. Current long-term treatments include transplanting patients with islet cells isolated from donor cadavers. This method requires finding at least two cadaver sources per patient and is reserved for those with advanced complications. As a result less than 10% of islet cell transplants are successful and they are eventually rejected within 1-5 years. Differentiating stem cells to become glucose responsive insulin secreting cells is a potential therapy for type 1 diabetics. We have shown that human embryonic stem (ES) cells can differentiate into insulin producing cells *in vitro* and have the ability to reverse the symptoms of type 1 diabetes when transplanted into STZ-treated mice. Cells were positive for pro-insulin as detected using a human specific antibody to pro-insulin and this response increased with the addition of glucose into the medium. We have differentiated ES cells into pancreatic precursors by adapting a modified Novacell protocol. *In vitro* results revealed expression of critical islet transcription factors including nkx6.1, pdx and mafA cells. We have transplanted them into STZ treated-NOD/SCID mice to determine the efficiency of engraftment, survival rate and insulin secretion rate. Diabetic animals were also able to regulate and maintain normal levels of glucose. Upon removal of the cells the diabetic symptoms returned including high blood glucose.

Poster Board Number: 1010

ALLOGENIC MURINE BONE MARROW MONONUCLEAR CELLS TRANSPLANTATION SUPPLEMENTED WITH ANTIOXIDANT COCKTAIL HAS CLINICAL RELEVANCE TO CURE DIABETES

Ambasta, Rashmi K.[1], Arivarasan, A.[1], Shaha, Harshit[1], Kumar, Pravir[2]

[1]Center for Medical Engineering, Vellore Institute of Technology, Vellore, India, Vellore, India, [2]Center for Medical Engineering, Tufts University School of Medicine, Boston and Vellore Institute of Technology, Vellore, India, Vellore, India

Diabetes is a rapidly growing health burden to the global society irrespective of race and region. However, researchers are involved in the exploration of effective clinical therapy for diabetes. Currently either regular medication or injections are only the solution to reduce the severity of diabetes. Moreover, stem cell transplantation has been tried to rejuvenate the atrophied pancreatic cells but this therapy has their own limitations in terms of identification and maintenance of specific stem cell markers in cell culture condition. Therefore, cell therapy using bone marrow (BM)-derived mononuclear cells (BMNCs) can be one of the best treatment options as it can differentiate into stem cell and also is capable of regenerating damaged pancreatic islet cells. We aim to investigate diabetes cure using BMNCs transplantation and antioxidant rich cocktail derived from natural products in a murine model. We are reporting for the first time that intravenous transplantation of BMNCs supplemented with anti-oxidant cocktail is a better way of transplantation therapy than the BMNCs transplantation alone. Interestingly, in this study, we have found the translocation of intravenously injected DiI stained BMNCs into the damaged pancreas and kidney. However we are further investigating the fate of DiI stained BMNCs in other organs. Surprisingly, one time administration of transplanted BMNCs conjugated with antioxidant cocktails to the pancreas attenuates the high blood sugar level to the normal range within a short period of time that consistently lasts for months after transplantation. Also, the health and longevity of the mice after transplantation were observed reasonable and no further complications have been seen. Hence, we conclude that allogenic murine bone marrow mononuclear cell transplantation supplemented with antioxidant cocktail has the potential to cure diabetes.

Poster Board Number: 1012

PANCREATIC DEVELOPMENTAL INTERMEDIATES DEMARCATED BY TRANSCRIPTIONAL PROFILING OF MOUSE PROGENITOR CELLS

Potter, Leah A.[1], Osipovich, Anna[1], Choi, Eunyoung[1], Gangula, Rama[1], Manduchi, Elisabetta[2], Kraus, Marine[3], Stoeckert, Christian[2], Grapin-Botton, Anne[3], Magnuson, Mark[1]

[1]Vanderbilt University, Nashville, TN, USA, [2]University of Pennsylvania, Philadelphia, PA, USA, [3]Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

A thorough understanding of developmental pathways is essential for both developing and assessing the veracity of protocols for directing the differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to specific cell fates. During mouse development, pancreatic endocrine cells form as the result of a sequential process that involves at least four developmental intermediates including 1) definitive endoderm, 2) the primitive gut tube, 3) pre-pancreatic endoderm, and 4) islet progenitor cells. These developmental intermediates are distinguished by the expression of stage-specific transcription factors. Prior studies to compare the gene

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expression profiles of these progenitor cells relied on both DNA microarrays for profiling and manual dissection methods for cell isolation, both of which diminish the value of the data obtained. To overcome the issue of cell purity, we have developed a series of fluorescently-tagged reporter alleles in mice (Sox17GFP-Cre, Pdx1Cerulean, Ptf1aCitrine, and Insm1GFP-Cre) that can be used to obtain genetically defined progenitor cell populations by fluorescence-activated cell sorting (FACS). Moreover, we have used these cell populations to perform RNA-seq to better quantify key aspects of the temporal, spatial and transcription factor-dependent gene expression in pancreatic or pre-pancreatic progenitor cells. Analysis of the data obtained to date has revealed multiple, stage-specific gene expression clusters thereby suggesting the involvement of additional transcription factors, cell-surface receptors and signaling pathways in key stages of pancreas development. Based on the quality of the results being generated, this strategy promises to provide a much higher resolution than currently exists for gene expression dynamics during early pancreas development.

Poster Board Number: 1014

TRANSPLANTATION OF INSULIN-PRODUCING CELLS IN ALGINATE BEADS GENERATED BY A SCALEABLE EMULSION AND INTERNAL GELATION PROCESS

Hoesli, Corinne A.[1], Kiang, Roger LJ[2], Mocinecová, Dušana[3], Speck, Madeleine[4], Jochek Moskova, Daniela[3], Donald, Christine[4], Lacík, Igor[3], Kieffer, Timothy J.[4], Piret, James M.[2]

[1]Chemical Engineering, Université Laval, Québec, QC, Canada, [2]Michael Smith Laboratories & Department of Chemical and Biological Engineering, University of British Columbia, Vancouver, BC, Canada, [3]Polymer Institute, Slovak Academy of Sciences, Bratislava, Slovakia, [4]Department of Cellular and Physiological Sciences & Department of Surgery, University of British Columbia, Vancouver, BC, Canada

Recent years have seen the development of more efficient methods to differentiate embryonic stem cells into insulin-producing cells. One major hurdle that remains for the use of these cells to treat diabetes is the anticipated requirement for immune suppression or immune isolation of these cells. We have recently developed a scaleable emulsion-based process to encapsulate pancreatic cells in alginate beads. The objective of the current work was to examine the immune protection conferred by these novel emulsion-generated beads. Compared to a standard extrusion-based process, the novel emulsion-based process allowed to generate alginate beads with higher *in vitro* stability and higher volumetric exclusion of antibody-sized dextrans. When βTC3 cells were transplanted into streptozotocin-induced allogeneic diabetic mice, a significant decrease in the blood glucose levels was seen within 2 days with the emulsion beads but not until >16 days with the extrusion beads. This was correlated with higher *in vivo / in vitro* DNA yields and lower graft-specific plasma immunoglobulin levels. These results suggest that the novel emulsion-generated alginate beads could provide improved graft immunoprotection. The emulsion process could be applied to other stem cell types to either manipulate their fate or to protect them from immune rejection in transplantation applications.

Poster Board Number: 1016

REVERSAL OF HYPERGLYCEMIA BY INSULIN-SECRETING CELLS DERIVED FROM RAT MULTIPOTENT ADULT PROGENITOR AND EXTRAEMBRYONIC ENDODERM PRECURSOR CELLS

Kumar, Anujith[1], Gysemans, Conny[1], Cai, Qing[1], Heremans, Yves[2], Esguerra, Camila[1], Jiménez-González, María[3], Mathieu, Chantal[4], Binas, Bert[5], Prosper, Felipe[3], Verfaillie, Catherine[1], Barajas, Miguel[3]

[1]Stem Cell Institute, KU Leuven, Leuven, Belgium, [2]Stem Cell Institute, University of Minnesota, Minneapolis, MN, USA, [3]Hematology and Cell Therapy and Foundation for Applied Medical Research, Division of Cancer, Clínica Universidad de Navarra, University of Navarra, Pamplona, Spain, [4]Laboratory for Experimental Medicine and Endocrinology, KU Leuven, Leuven, Belgium, [5]Division of Molecular & Life Science, College of Science and Technology, Hanyang University, South Korea, Korea, Republic of

Type I diabetes mellitus is a devastating disease due mainly to impaired insulin production by β-cells, which can only be cured by transplatation of new β-cells. In the present study, we report the efficient generation of insulin-secreting cells from two cell sources: Multipotent adult progenitor cells (MAPC) derived from rat bone marrow and Extraembryonic endoderm Precursor (Xen-P) cell lines obtained directly from rat under MAPC conditions. MAPC as well as Xen-P cells isolated from Blastocysts under MAPC conditions have the potential to differentiate *in vitro* to cells with endothelial, smooth muscle, hepatocyte-like and neuroprogenitor cell-like cells. The three step differentiation protocol designed in this study towards pancreatic lineage involves initial endoderm commitment, pancreatic epithelial expansion and later cell maturation. After 21 days of differentiation, 20-25% of the differentiated cells expressed Pdx1 and there was a significant upregulation of insulin gene expression. The glucose responsiveness of these cells was demonstrated by combined incubation with glucose and the Calcium channel blocker, Nifedipine, or incubation with the insulin agonist, Carbachol. As a proof of therapeutic significance, we demonstrate that implantation of ±5x105 MAPC and Xen-P cell-derived insulin producing cells under the kidney capsule reverts streptozotocin-induced hyperglycemia in nude mice, with normalization of the glucose tolerance test. Hyperglycemia recurred following graft removal. Analysis of the graft demonstrated expression of critical beta-cell transcription factors as well as insulin and C-Peptide. The ability of MAPC isolated from BM or its physiological equivalent isolated from balstocyst, to differentiate to β-cell like cells may serve as a useful tool to gain insight into signals that govern β-cell differentiation. This protocol could also serve to differentiate other pluripotent stem cells yielding a renewable source of islets for diabetes cell-replacement therapies.

Poster Board Number: 1018

NGN3 PROGRAMMING OF ENDOCRINE PANCREAS DIFFERENTIATION

Gibson, Jason D., Chung, Kyung-min, Nelson, Craig E.

Molecular and Cell Biology, University of Connecticut, Storrs, CT, USA

The ability to generate therapeutic cell types from stem cells is at the heart of many envisioned procedures in the promising field of regenerative medicine. Extensive research investigating endoderm development and pancreatic organogenesis has resulted in a comprehensive understanding of key signaling events driving pancreas development, detailed transcriptional profiles of pancreatic progenitor cells, and the developmental trajectories of hormone-producing cells in the endocrine pancreas. Exploiting this knowledge, many labs have shown stepwise guided differentiation of embryonic stem (ES) cells can effectively generate pancreas-like lineages, including insulin-producing beta-like (β) cells. However, these cells have not proven robust in trans-

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plantation or functional characterization assays, suggesting that while they express many β-cell markers, they lack critical pancreatic functions. A better understanding of the transcriptional regulatory network guiding cell type specification will help identify how these critical functions are controlled and help improve the directed differentiation of β-cells from stem cells. In these studies, we examine the role of enhanced neurogenin 3 (Ngn3) expression in promoting ES cell differentiation to endocrine pancreas cell types.The transcription factor Ngn3 is a crucial regulator of endocrine cell specification and is required for promoting pancreatic islet maturation including the development of insulin-producing β-cells and the maintenance of islet function within the mature pancreas. Our research has shown that Ngn3 can direct ES cell differentiation toward the endocrine pancreas lineage, and highlights the importance of timed induction of this protein for proper islet differentiation. Our present data indicates that definitive endoderm progenitor populations derived from ES cells are responsive to Ngn3 signaling throughout stepwise directed differentiation protocols but earlier stages of ES cell differentiation are less receptive to Ngn3 activity. Studies pursued using an inducible transgenic system will provide a tractable experimental platform to study cellular cross-talk between Ngn3-positive and Ngn3-negative cells during pancreas differentiation.

Poster Board Number: 1020

A SMALL MOLECULE SCREENING PLATFORM TO IDENTIFY COMPOUNDS THAT INCREASE FUNCTIONAL BETA CELLS FROM IPSCS

Bright, Jessica, Cooper, Bonnie, Wang, Weidong, Wolfson, Dmitri, Strulovici, Berta, Irion, Stefan

iPierian, South San Francisco, CA, USA

The absolute or relative lack of insulin secreting pancreatic β cells plays the central role in the development of diabetes Type 1 and Type 2, respectively. The discovery of the insulin hormone, its isolation and synthetic production have impacted the clinical course of the disease dramatically. Treatment with synthetic insulin however requires tight monitoring of glucose levels and precisely coordinated insulin injections. Many laboratories have thus established research projects to generate β cells from human pluripotent stem cells. Such β cells could be used for cell replacement therapies, for drug discovery efforts and to study the basic biology of the islets of langerhans. However current differentiation protocols are limited to inefficient methods that yield immature β cells in a heterogeneous gemisch of other islet and other non-pancreatic cells. To overcome these limitations we established a screening platform for small molecules that could complement or replace current methods. While many labs, including ours, are very efficient in generating PDX1pos pancreatic progenitors, a major obstacle is the subsequent differentiation into hormone positive, functionally mature cells. We consequently focused on this stage of pancreatic differentiation. To do so we first employed a serum free differentiation protocol to generate definitive endoderm progenitors from multiple human induced pluripotent stem cell lines (iPSCs). These progenitors were subsequently specified to pancreatic progenitors using retinoic acid, Dorsomorphin, FGF10 and inhibitors of the sonic hedgehog pathway. After 15 days of differentiation these cells were passaged and allowed to mature until day 35, at which about 5% of the cells expressed C-PEPTIDE, as determined by flow cytometry. In a C-PEPTIDE ELISA these cells responded to glucose, albeit at various levels, indicating their immature status. To address these restrictions we began to establish a small molecule screen for inducers of mature and functional β cells. We assumed that our induction of PDX1 progenitors was efficient and provided a suitable starting material for these studies. We plated pancreatic progenitors in 384-well plates and exposed them to a subset of our chemical library at two different time points (3000 compounds, all at 10uM). After a single treatment cells were further cultured and analyzed for the expression of C-PEPTIDE by immunocytochemistry using an MDS automated confocal imaging platform. We then established a custom image analysis algorithm that is capable of detecting C-PEPTIDEpos cells with a sensitivity of 82% and a specificity of 87%, which is en par with trained individuals. To this date we used this platform to validate our approach and a handful of candidate compounds have been identified that increase C-PEPTIDEpos cells above the median. Their ability to routinely increase the frequency and maturity of C-PEPTIDEpos cells is currently being evaluated. SUMMARY: We established a small molecule screen to increase the number and function of iPSCs derived β cells. Assay validation with a subset of our chemical library was successful and a few selected compounds will be tested in secondary assays, while the results from this pilot experiment will help us to improve the current assay.

LIVER CELLS

Poster Board Number: 1022

DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO FUNCTIONAL HEPATOCYTES USING THE COMBINATORIAL CELL CULTURE PLATFORM COMBICULT™

Jeyakumar, Jey, Tarunina, Marina, Johnson, Christopher, Ramathas, Vidya, Saw, Dennis, Hook, Lilian, Choo, Yen

Plasticell Ltd, London, United Kingdom

Generating functional hepatocytes from human embryonic stem (hES) cells remains a major challenge due to inefficient differentiation protocols, characterised by low yields, cellular heterogeneity and lack of functional attributes typical of primary hepatocytes. Here we describe the discovery of several highly efficient serum-free hepatic differentiation protocols using a Combinatorial Cell Culture technology called CombiCult™. 3,375 pathways designed to mimic human embryonic liver development were screened using this high throughput technology. hES cells were cultured on microcarrier beads and shuffled through 3,375 combinations of cell culture conditions, containing growth factors and/or small molecules over 21 day period. As the beads pass through each of the culture conditions they are marked with a unique set of tags, enabling identification of the cell culture history for each of the beads bearing hepatic cells. Albumin and/or CYP3A4 expression was used as a marker to identify successful hepatic differentiation. Through this method, a number of highly efficient protocols were identified and subsequently validated in a monolayer culture system and characterised at cellular and functional levels. These hES-derived hepatic cells exhibit the morphology, marker expression, and function of authentic hepatic cells, promising to be a valuable tool for regenerative medicine and drug discovery applications.

Poster Board Number: 1024

MODELING STEATOSIS AND STEATOHEPATITIS USING HUMAN INDUCED PLURIPOTENT STEM CELLS

Jozefczuk, Justyna[1], Loibner, Martina[2], Ummanni, Ramesh[3], Korf, Ulrike[3], Zatloukal, Kurt[2], Lehrach, Hans[1], Adjaye, James[1]

[1]Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Berlin, Germany, [2]Institute of Pathology, Medical University of Graz, Graz, Austria, [3]Division of Molecular Genome Analysis, Deutsche Krebsforschungszentrum (DKFZ), Heidelberg, Germany

Non-alcoholic fatty liver disease comprises a broad spectrum of disease states ranging from simple steatosis (S) to steatohepatitis (SH). In contrast to S which has a good prognosis and can be cured relatively easily, SH is associated with alterations of a variety of cellular functions including increased cellular stress that lead to serious hepatocyte injury and may result in liver cirrhosis and development of a lethal cancer - hepatocellular carcinoma. The major unsolved question is what genetic background and molecular

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mechanisms result in the development of SH followed by cirrhosis and cancer as compared to other individuals who develop only steatosis. So far one of the biggest obstacles impeding studying the susceptibility to SH and its progression to hepatocellular carcinoma is the lack of a simplified *in vitro* system which would allow reproducible testing of the influence of various genetic and environmental factors on the etiology of this lethal disease. The generation of patient-specific iPS cells followed by their differentiation into hepatocytes enables the analysis of steatosis and steatohepatitis in a reproducible, standarized and high throughput manner. The main goals of our study are the generation of a system level dataset to enable the studying of the molecular mechanisms underlying the etiology of both diseases followed by the development of a computational model quantitatively explaining the response of S and SH hepatocytes towards various stimuli mimicking key aspects of environmental exposures potentially increasing the risk of developing SH (high doses of fatty acids, glucose, insulin, reactive oxygen species). The entire dataset will be comprised of transcript, protein and metabolic profiling of iPS-derived hepatocytes obtained from 15 patients-specific fibroblasts cell lines. The main biological question to be addressed is the comparison of lipid metabolic pathways between S, SH and non-liver disease iPS-derived hepatocytes with a special emphasis on the relationship between fatty acid metabolism, insulin resistance and intra-hepatic triglycerides content. Another key question is whether iPS-derived hepatocytes from obese patients have the inherited ability to develop the known features of the cellular pathology, such as the accumulation of intracellular triglycerides. Here we present initial results on dermal fibroblast derivation and characterisation of iPS cells from both steatosis and non-liver disease patients as controls. Followed by the differentiation into hepatocytes, then comparative transcriptome and reverse phase protein array-based analysis of insulin signalling and drug metabolism pathways. This initial dataset provides the foundation to address the influence of genetic background and also identify both novel biomarkers for metabolic risk and unique targets for therapy.

Poster Board Number: 1026

METABOLICALLY FUNCTIONING HEPATOCYTES ARE DIFFERENTIATED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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Human induced pluripotent stem cells (hiPSC) hold great potential for use in regenerative medicine, novel drug development, and disease progression/developmental studies. One of the key concerns for employing iPSC for these applications is their ability to be fully differentiated. The differentiation of the hiPSC line, IMR90-4, was initiated by first directing the cells towards a very pure population of definitive endoderm, then further towards hepatocytes. The metabolic function of hiPSC-derived hepatocytes (hiH) was characterized by the expression of metabolizing enzymes, and metabolic profiling of the drug bufuralol (BF). The differentiated hiPSC showed 84% of cells positive for albumin, indicating a highly efficient process for generating a relatively homogenous population of hepatocytes. Metabolizing enzymes, phase I enzymes, such as CYP 1A1, 2C9, 2C19, 2D6, 3A4, 7A1, and phase II enzymes, such as GSTA1-1, GSTP1-1, UGT1A1, 1A3, 1A10, UGT2B7, were expressed in hiH, as determined by Western blot and RT-PCR. Moreover, transporters, phase III proteins such as MRP1 and OATP2 were also expressed in hiH, as determined by immunochemistry. Some important nuclear regulators, critical in regulating the expression of metabolizing enzymes, including CAR, AhR, CPR, and PXR, were expressed in hiH, as assessed by immunochemistry. The cellular uptake and excretion of Indocyanine Green (ICG) is a unique characteristic of hepatocytes, and it was observed in our hiH in a very high percentage of cells. The excretion of ICG began 1 hour after the removal of ICG, and most of the ICG was excreted within 3.5 hours, and it almost completely disappeared by 24 hours, indicating that a functional biotransforming system was generated by our hiH. To further assess the biotransformation system, we employed ultraperformance liquid chromatography-tandem mass spectrometry technology for BF metabolic profiling and metabolism. Predictive multiple reaction monitoring (MRM) is the most sensitive approach in metabolite identification. With the evidence of the same MRM transitions, LC retention time, MS/MS fragmentation patterns, and time-course effects of these secondary metabolites, we found 4 secondary metabolites of BF from 2 different oxidations, dehydrogenation, ketone formation, or potential methylation in the phase I stage, and 3 secondary metabolites of BF from glucuonidation, conjugations of glucose and glutathione in the phase II stage, in both hiH and freshly isolated human primary hepatocytes (hPH). Thus, we have successfully identified seven major metabolic pathways of BF in hiH, which are the same as those in hPH. This is the first time that hiH have been shown to develop full metabolic function as do the hPH. After transplantation in MPSVII SCID mice, Fah-/-/Rag2-/-/Il2rg-/- mice and NOD/SCID mice through splenic injection, our hiH engrafted in mouse livers and human liver-specific protein products were detected in the mouse livers and in the mouse serum 2-4 weeks after transplantation. These results demonstrate that functioning patient-or disease-specific hepatocytes could be generated for cell-based therapeutics, as well as for pharmacology and toxicology studies.

Poster Board Number: 1028

INDUCED PLURIPOTENT STEM CELL MODELING OF HUMAN HEREDITARY AMYLOIDOSIS

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Amyloidosis refers to a group of rare diseases of systemic extracellular deposition of misfolded fibrillar proteins, which leads to organ failure and death. The most common form of familial amyloidosis (AF) across the globe is due to diverse mutations in serum transthyretin (TTR). Familial transthyretin amyloidosis (ATTR) is a lethal, autosomal dominant disease mediated by more than 100 distinct mutations in the transthyretin gene. Predominantly synthesized in the liver, mutant TTR protein deposits in multiple extrahepatic tissues, including the peripheral nervous system, heart, and the gastrointestinal tract disrupting organ function. ATTR represents a tremendous unmet medical need with significant morbidity and mortality. Liver transplantation is the only proven treatment option, yet only about one third of patients are candidates for this invasive procedure. As with most amyloid diseases, the detailed molecular mechanisms involved in the development of ATTR are not fully understood, in part due to the lack of an appropriate model and the inability to study the disease in the appropriate cell lineage with the appropriate genetic background. The recent discovery that pluripotent cells similar to embryonic stem (ES) cells can be generated by reprogramming somatic fibroblasts provides an unprecedented opportunity to model human disease and to develop regenerative therapies for genetic diseases. Like ES cells, the broad differentiation repertoire of these so called induced Pluripotent Stem Cells (iPSC) highlights their potential to form any desired somatic cell type, including hepatocytes. In contrast to ES cells, iPSC are genetically identical to the individual from whom they are derived, raising the prospect of utilizing iPSC for autologous cell-based therapies without risk of rejection. Diseases that affect the liver represent excellent targets for iPSC-based studies. A critical issue concerning the use of human iPSC, however, is whether they can generate terminally mature progeny with normal function. This point is highlighted in the study of liver specification when

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one wishes to recapitulate *in vivo* ontogeny in an *in vitro* system in which the sequential development of primitive and definitive characteristics are accompanied by the appropriate, stage-specific expression of hepatic genes. We have developed a novel, excisable polycistronic vector (STEMCCA) for the generation of 'clinical grade' human iPSC free of exogenous transgenes. Using this technology, we have created the first known ATTR-disease specific human iPSC lines and demonstrate the efficient derivation of definitive endoderm and liver-specified progeny from these cells. Our directed differentiation protocol allows us to direct greater than 65% of our human iPSC-derived endoderm progenitors into hepatocyte progenitors, defined as cells co-expressing alpha fetoprotein (AFP), Foxa1, HNF4A, and albumin. Importantly for the recapitulation of the ATTR disease phenotype, both normal and mutant forms of TTR are also detectable using a combination of isoelectric focusing (IEF) and mass spectrometric (MS) analyses. Lastly, we demonstrate small molecule inhibition of aberrant TTR production further validating the value of IPSC-based disease modeling. The long-term goals of this work are to better understand the molecular mechanisms underlying the pathogenesis of hereditary amyloidosis and to develop novel therapies for the treatment of the disease.

Poster Board Number: 1030

HIGH PURITY SCALABLE PRODUCTION OF HEPATOCYTES FROM HUMAN EMBRYONIC STEM CELLS

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There is significant demand for human hepatocytes for use in high throughput drug screening, drug development and predictive toxicology. Human embryonic stem cell (hESC)-derived hepatocytes present several advantages over primary cell hepatocytes of human cadaveric origin currently on the market. In addition to the ability to produce cells on demand and in virtually unlimited quantities, hESC-derived hepatocytes present a relevant model for human ADME/toxicology studies. We have developed a fast, scalable method to directly differentiate hESCs into the high purity hepatocytes using well-defined media, with minimal animal components, resulting in mature functional hepatocytes within twenty-four days. The cells express appropriate immunocytochemical markers and possess functional activities of mature hepatocytes, such a glycogen storage, albumin production and indocyanine green dye uptake. In addition, the induction of CYP activity is comparable to that of human primary hepatocytes. California Stem Cell's hESC-derived hepatocytes will be available in 96-well and 384-well plate formats that are suitable for high throughput screening assays for drug development and predictive toxicology.

Poster Board Number: 1032

HUMAN STEM/PROGENITOR CELL-DERIVED HEPATOCYTES PROVIDES ROBUST MODEL FOR DRUG METABOLISM

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Human hepatocytes are used routinely throughout Drug discovery for drug metabolism studies, transporter studies and toxicity screens. The quality and availability of the gold standard, primary fresh and cryopreserved human hepatocytes is highly variable and the supply is limited. This results in the need to employ other hepatic models such as established human hepatoma cell lines, HepaRG and HepG2, that are more scalable and subcellular fractions, microsomes, to offer the opportunity of having many human models that cover most hepatic functions. Animal hepatocytes are more readily available and physiologically relevant, but are often not predictive of findings in humans. Consequently, since a single model doesn't possess all the functions of *in vivo* hepatocytes, multiple hepatic model systems have become necessary. However, due to the limitations of the many different hepatic models, there is still a need to develop a better model system that has more complete hepatic function. Here we provide data to demonstrate that human stem/progenitor cell-derived hepatocytes may offer a better model system to meet that need. We have developed a hepatic model system that includes human stem/progenitor cells, novel formulations of lineage stage specific media for expansion and differentiation of stem/progenitor cells and maintenance of mature hepatocytes as well as biomatrix isolated from decellularized liver. Human stem/progenitor cells differentiate into mature hepatocytes within a few days and maintain functions for months. Primary hepatocytes plated on the biomatrix in maintenance media maintain function longer than when plated on collagen I. This data will demonstrate that our pre-clinical reproducible, scalable hepatic model will provide hepatocytes that are more functional, more physiologically relevant and more predictive of clinical outcome than current models, therefore reducing attrition of drugs that enter the clinic.

Poster Board Number: 1034

A FEEDER-FREE AND SERUM-FREE PRODUCTION OF MULTI-FUNCTIONAL MATURE HEPATOCYTES WITH ELECTRON MICROSCOPICALLY VALID MORPHOLOGIES: TOWARDS AN ESTABLISHMENT OF THE GLOBAL STANDARD FOR HUMAN ES/IPS-BASED DRUG DISCOVERY TOOLS

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<OBJECTIVES> Hepatotoxicity is a common cause of failure in drug discovery. It is thus particularly important to understand the metabolism and hepatotoxicity of the drugs in the early phase of pharmaceutical development. Although primary human hepatocytes and purified human liver microsomes have been providing excellent systems, they suffer from lot variation, high cost and expansion incapability. To provide more serviceable systems, we performed hepatic differentiation of human ES/iPS cells under a feeder-free and serum-free condition. <METHODS> One line of human ES cells (khES-1), four lines of retroviral vector-based iPS cells (253G1, 201B7, #25 and #40) and one line of Sendai virus vector-based, transgene-free iPS cells (SeV5) were subjected to a 5-tired differentiation system* mimicking *in vivo* hepatic development: Step 1 (Day 0); endoderm induction (first half) by Activin A and Wnt-3A, Step 2 (Day 1); endoderm induction (second half) by Activin A, Step 3 (Day 2 ~ 6); hepatic initiation by FGF-2, BMP-4 and SHH, Step 4 (Day 7 ~ 11); hepatic maturation (first half) by HGF, FGF-2 and BMP-4, Step 5 (Day 12 ~ 30); hepatic maturation (second half) by OSM and dexamethasone. Maturation of human ES/iPS-derived hepatocytes was evaluated by message and protein expressions, glycogen storage, indocyanine green (ICG)-uptaking/releasing capacities, cytochrome P450 (Cyp) activities and electron microscopic (EM) observations. <RESULTS> In the human ES-derived and all iPS-derived cells, the expressions of α-fetoprotein, albumin, α1 anti-trypsin were confirmed by RT-PCR, Western blotting and immunostaining. Majority of the cells (> 80 %) were positive for PAS-staining (glycogen storage), and one third of the cells demonstrated ICG-

uptaking/releasing capacities. Several fold Increments in Cyp 3A4 activities were detected in the human ES-derived and all iPS-derived hepatocytes compared to their immature counterparts. Moreover, Cyp 3A4 activities were significantly induced by drug treatments (high dose dexamethasone and rifampicin). EM observation further confirmed the maturation of ES/iPS-derived hepatocytes: they showed rich microvillous projections characteristic to "Space of Disse", hepatocyte-specific, rosset-forming glycogen granules called "α-particles" and "bile canaliculi" surrounded with tight junctions (proximal) and desmosomes (distal). Interesting, minor bile duct components with low-height microvilli on lumenal sides and basement membranes on the opposite sides were also detected. <DISCUSSSION> Our method has enabled the production of multi-functional mature hepatocytes with electron microscopically valid morphologies from human ES/iPS cells. Our system can be applied to wide varieties of human iPS lines including Sendai virus vector-based, transgene-free iPS cells. Existence of minor bile duct components suggests co-generation of hepatic stem cells. Currently, we are establishing a feasible method for the assessment of hepatotoxicity of the drugs by measuring concentrations of liver-specific enzymes in culture supernatants. Our method will greatly contribute to an establishment of the global standard for human ES/iPS-derived drug discovery tools. <Acknowledgements> Authors greatly thank Professor Yamanaka at Center for iPS Cell Research and Application, Kyoto University, Japan, for generously providing the hiPSC lines (253G1 and 201B7).

* This system is currently Japanese patent pending (Tokugan 2011-019103).

Poster Board Number: 1036

HEPATIC PROGENITORS CAN BE PURIFIED UPON DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

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Hepatocyte transplantation is now an alternative to liver transplantation for the treatment of liver metabolic diseases. However this approach is restricted by the lack of donors and the limited number of hepatocytes since these cells are difficult to cryopreserve and cannot be expanded *in vitro*. Isolation and amplification of hepatic progenitors should provide an alternative solution. Generation of hepatocyte-like cells from human embryonic stem (hES) cells have already been reported using undefined matrices and animal products in diverse culture conditions, all of which generating mixed cell populations. Recently we developed chemically defined differentiation conditions, which recapitulate key stages of liver development, to generate hepatic progenitors representing about 60% of the cell population. We then developed a purification approach by fluorescent activated cell sorting (FACS) using a self inactivated lentivector encoding eGFP driven by the human Apolipoprotein A-II hepatic specific promoter (APOA-II). Furthermore, we designed a novel strategy to prevent vectors integration into the genomic DNA. Conditions yielding to transduction of 70% of undifferentiated cells at MOI 30 with an EF1α-eGFP vector were used. Southern blot analyses demonstrated that the genomic DNA from transduced cells was devoid of provirus DNA under our conditions. To insure the specificity of APOA-II-eGFP, its expression was followed along hES cells differentiation. While eGFP expression was detected in less than 1% of cells at day 3, and in about 2% of cells at day 8, it rose to 18% at day 14 and reached 35-39% at day 16 of differentiation, which was in correlation with endogenous APOA-II expression. Transduced cells were then purified at day 16 by FACS, yielding 99% of eGFP positive cells. Hepatic progenitor markers such as cytokeratine-19 (CK19) and α-fetoprotein (AFP) were found co-expressed in most of cells as assessed by immunocytochemistry, demonstrating that sorted cells were bipotential hepatic progenitors. Quantification by quantitative PCR and flow cytometry analysis as well as phenotypic characterization of differentiated sorted cells is currently performed.

Our data indicate that hepatic progenitors can be purified using the APOA-II hepatic specific promoter carried by lentivectors and suggest a potential use of these progenitors for clinical applications.

Poster Board Number: 1038

HIGH HEPATIC POTENTIALS IN MINI-SCALED FLOW-CULTURE SYSTEM OF MURINE OR HUMAN ES/IPS CELL-DERIVED HEPATIC TISSUE CONSISTING OF HEPATOCYTES AND ENDOTHELIAL CELLS

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Hepatocyte has multiple specific functions in a cell, different from other cells having a single specific function. In order to establish an *in vitro* hepatocyte culture or artificial liver system being similar to the *in vivo* liver, it is necessary to maintain all of the multiple functions. However, it has been still developing materials, medium conditions, and apparatuses for the culture of the primary hepatocytes. On the other hand, there are so many reports that hepatocytes were obtained from the differentiation of ES or iPS cells. However, it is clear to be difficult to maintain the multiple hepatic functions in the single culture of ES or iPS cell-derived hepatocytes because it has been still difficult to culture primary hepatocytes for a long period. We consider that ES cells and iPS cells give promises in constructing *in vitro* liver models. Our purpose is to establish a micro-sized flow-culture system of murine or human ES/iPS cell-derived hepatic tissue being close to *in vivo* liver. An *in vitro* system for liver organogenesis from murine ES/iPS cells was established in our previous studies. These hepatic tissues consisted of hepatocytes and endothelial cells, which had higher hepatic function than that of primary cultures of murine embryo and adult hepatocytes. The result indicated that hepatic tissue reconstruction including parenchymal cells, as hepatocytes, and non-parenchymal cells, as endothelial cells, should be necessary for maintaining hepatic functions *in vitro*. In the case of human liver tissue, firstly, immortalized human umbilical vein endothelial cells, HUVECs, were culture on some biomaterials to form the tube network structure. Several differentiation stage cells from the human iPS cells on the hepatic lineage induced by addition of specific growth factors were co-cultured on the immortalized HUVEC tube network. In the above culture, hepatic lineage cells were migrated to the network to form hepatic sinusoid-like structure. This human iPS cell-derived sinusoidal culture had much higher hepatic functions such as ammonia metabolism and cytochrome P450 activities compared to those of single culture of human iPS cell-derived hepatocytes. Human ES cells are trying to be differentiated by same way now. We also invented mini-scaled flow-culture system in which pH and dissolved oxygen concentration could be monitored. These murine or human ES/iPS cell-derived hepatic tissues were cultured and used for drug metabolism assays in this flow culture system. These hepatic tissues could have higher hepatic potentials in the culture for a long period. In future, we expect that our hepatic tissue culture system could be useful as an application of evaluating drug metabolism and hepatotoxicity in the development of new drugs and chemicals.

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Poster Board Number: 1040

ALTERNATIVE MOUSE PLURIPOTENT STEM CELLS AS A SOURCE OF HEPATIC CELLS

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Recently there have been scientific reports that have described the generation of pluripotent stem cells from the mammalian germ-line. Parthenogenetic activation of female oocytes can give rise to blastocysts, whose inner cell mass can be isolated and propagated as parthenogenetic embryonic stem cells (pESCs). Murine testis-derived pluripotent germ-line stem (gPSCs) cells, obtained from adult mouse testes through culture of unipotent spermatogonial stem cells under ES cell conditions, has also been recently described. The pESCs and gPSCs show expression of pluripotency markers, at levels comparable to embryonic stem cells (ESCs). In our present study we investigate the hepatic differentiation potential of these alternative sources of pluripotent stem cells. We have previously reported the use of an embryoid body formation-based protocol to derive hepatic progenitors from the gPSCs and the pESCs. However, this protocol lacked efficiency in generating higher number of hepatic cells with a mature phenotype that were suitable for further transplantation experiments. We have also previously reported a cytokine based differentiation protocol which mimics the *in vivo* embryonic development of the liver. Modification of this cytokine-based protocol was able to generate higher numbers of hepatocyte-like cells, with expression of hepatic markers comparable to Hepa 1-6 cells. Activin A treatment of pESCs and gPSCs grown as a monolayer in serum-free differentiation (SFD) medium, initiated the induction of early endoderm. Further treatment of the cells with Bone Morphogenic Protein 4 (BMP4) and basic Fibroblast Growth Factor (bFGF) directed the cells towards the definitive endoderm. The cells were then cultured in Hepatocyte Culture Medium (HCM) along with Oncostatin M (OSM) and human Hepatocyte Growth Factor (hHGF), in order to induce hepatic cell maturation. Albumin producing cells were selected using an Albumin-Neomycin vector and then transplanted into the FAH knockout mouse to check for engraftment and proliferation. Our study hence provides an insight into the use of germ-line derived hepatocytes for cell therapy in treatment of liver disorders.

Poster Board Number: 1042

ISOLATION AND CHARACTERIZATION OF A UNIQUE MOUSE GALLBLADDER STEM CELL POPULATION

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The identification and characterization of biliary stem cells has great importance for basic biology and cell-based therapy. The biliary tree is divided into intra- and extrahepatic systems. The latter consists of the gallbladder, cystic duct, common bile duct and pancreatic duct. Recent data have shown that intra- and extrahepatic biliary systems develop from separate progenitors implying that adult intra- and extrahepatic bile duct cells would be different. However, there are few reports regarding their similarities or differences. In addition, there is currently a paucity of data regarding extrahepatic bile duct stem cells. Here we report the identification and characterization of an EpCAM+CD49fhi sub-population of gallbladder cells with stem cell characteristics. We also show that these gallbladder stem cells have a unique phenotypic and expression profile compared to intrahepatic bile duct cells. Limiting Dilution Analyses from primary gallbladder indicate that EpCAM+CD49fhi cells are enriched in colony forming cells or stem cells compared to EpCAM+CD49flo cells (Exp1: 1/15 and 1/71; Exp2: 1/4 and 1/62). These data were confirmed by index sorts where ~92% of the single-cell derived colonies originated from EpCAM+CD49fhi cells. EpCAM+CD49fhi cells expand *in vitro* long-term (>passage 20) and exhibit single cell clonogenic expansion. We developed a matrigel differentiation assay in which EpCAM+CD49fhi cells exhibit organotypic morphogenesis recapitulating both ultrastructure and transport functions of primary gallbladder. We also observed similar morphogenesis when EpCAM+CD49fhi cells were injected into the subcutaneous space of recipient mice. These data demonstrate that EpCAM+CD49fhi cells possess stem cell characteristics of self-renewal and lineage commitment. We found expression of CD49f, CD49e, CD81, CD26, CD54 and CD166 to be different between primary intrahepatic and gallbladder cells by flow cytometry, indicating that they might be different. The transcriptomes of these cells were compared to expanded EpCAM+CD49fhi cells by cDNA microarrays. We found differences in the expression of key genes such as cytochrome P450, mucin3, solute carrier family, aquaporin1, interferon-regulated genes and IL-18. These data indicate that EpCAM+CD49fhi cells and intrahepatic bile duct cells have distinct transcriptomes and might have different functions. This is the first study to prospectively isolate and characterize stem cells from the adult mouse gallbladder. Furthermore, a major obstacle to studying intrahepatic bile duct cells has been a lack of appropriate expansion assays for the cells. The *in vitro* expansion assay and matrigel-based morphogenesis assay serve as technically facile and interchangeable tools to study bile duct biology. We postulate that these assays are generalizable for the study of bile duct biology, and would have large impact on the etiology of diseases such as biliary atresia.

Poster Board Number: 1044

PROSPECTIVE ISOLATION AND EXPANSION OF HEPATOBLASTS DURING EARLY-FETAL LIVER DEVELOPMENT IN MICE

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As the initial event of liver development, stem cells or progenitor cells in the liver bud, called hepatoblasts, proliferate and migrate into septum transversum mesenchyme at around embryonic day 9 (E9) in mice. Hepatoblasts are considered as somatic stem/progenitor cells in the fetal livers, because they have dual capacities of high proliferative potential and multi-lineage differentiation into both hepatocytes (hepatic parenchymal cells) and cholangiocytes. Several groups have analyzed hepatoblasts derived from mouse E11.5 to E14.5 mid-fetal livers. Previously, we have shown that a sorted individual cell derived from mid-fetal liver gives rise to a relatively large colony in the growth factor-supplemented culture on extracellular matrix-coated dishes. Dlk, E-cadherin, Liv2, CD13, and CD133 were identified as cell surface markers for mid-fetal hepatoblasts. This conventional culture system and the reclone-sorting culture have demonstrated that hepatoblasts in mid-fetal livers have self-renewal capacity and bi-potency to differentiate. In contrast to mid-fetal hepatoblasts, few studies have been done with epithelial cells found in early-fetal (E9.5 and 10.5) liver buds. The characteristics of these cells remain largely unknown, because no suitable culture system has been established. In this study, we found that cells expressing CD13, Dlk, and Liv2 exist during early- to mid-fetal liver development. Single CD13+Dlk+ cells purified from embryonic day 11.5 and 13.5 mouse mid-fetal livers, but not those purified from embryonic day 9.5 and 10.5 mouse early-fetal livers, formed individual large colonies in the conventional culture on collagen type-I coated dishes. Thus, we established a new co-culture system using mouse embryonic fibroblasts (MEF) as feeder cells for

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in vitro analysis of these cells. When E9.5 and E10.5 CD13+Dlk+ cells were co-cultured with MEF, several large colonies formed and consisted of both albumin-positive hepatocytic cells and cytokeratin 19-positive cholangiocytic cells, indicating that early-fetal CD13+Dlk+ cells can function as fetal hepatoblasts. Interestingly, when E9.5 CD13+Dlk+ cells were cultured on collagen type I, small and large colonies were detected only when MEF-conditioned media were used. Fresh medium not conditioned with MEF did not support colony formation on collagen-coated dishes. These data suggest that expansion of early-fetal hepatoblasts was partly supported by soluble factors derived from MEF. Inhibition of Rock or non-muscle myosin II (the downstream component of Rock) signaling was necessary for effective expansion of E9.5 CD13+Dlk+ cells. In sorted CD13+Dlk+ cells, expression of the hepatic marker genes albumin and α-fetoprotein was increased during fetal liver development. Expression of cytokeratin19 and Sox17, endodermal progenitor cell markers, was highest at embryonic day 9.5 but decreased dramatically thereafter. Collectively, our study indicated that CD13 and Dlk are expressed on hepatoblasts during early- to mid-fetal liver development and that expansion of early-fetal hepatoblasts requires the interaction with mesenchymal cells and the inhibition of Rock-myosin II signal pathway.

Poster Board Number: 1046

FUNCTIONAL IMPROVEMENT OF HEPATOCYTES CULTURED ON NANOFIBER-BASED 3D (NANEXTM) SCAFFOLD

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Hepatocyte in primary culture expresses various differentiated liver-specific functions *in vitro* for a certain period of time. However, it is generally recognized that human hepatocytes cultured in conventional 2D plastic culture dishes (TCPS) retain most of their functions only for a short period of time. A large-scale high-density hepatocyte culture, which maintains long-term albumin secretion, is expected to have many clinical and industrial applications, including a bio-artificial liver. In the present study, we evaluated the functional improvement of human hepatocytes cultured on nanofiber-based 3D (NANEXTM) scaffold. We assessed the proliferation, albumin secretion, and changes in cell morphology of the hepatic cell line HepG2 on NANEXTM surface. The results indicated that the human HepG2 cells cultured on nanofiber surface showed slower proliferation with increased albumin production for the first week. The Scanning Electron Microscope (SEM) analysis of HepG2 cells cultured on NANEXTM surface showed a tight aggregate of cells indicating an enhanced cell attachment in nanofiber surface as compared to 2D TCPS culture. Hepatocytes also exhibited polarized shape morphology when cultured in 3D nanofiber while cells spread more completely on TCPS 2D culture and developed flattened cell morphology. Taken together, our preliminary results indicate that nanofiber-based hepatocyte cultures exhibit better *in vivo*-like response than cells cultured in 2D. Our data suggest that the NANEXTM culture system may be a promising approach for several potential applications involving hepatocytes such as primary hepatocyte and liver stem cell *ex vivo* expansion, cell-based drug screening, toxicity testing, and/or liver regeneration.

Poster Board Number: 1048

A NOVEL MARKER FOR ENDODERMAL PROGENITOR CELLS IN TISSUE REPAIR AND TRANSFORMATION

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Analysis of tissue renewal and regeneration depends critically on the availability of markers for prospective isolation of stem and progenitor cells. Using the monoclonal antibody GCTM-5, we have identified a novel cell surface marker whose expression appears to be restricted to human tissues of endodermal origin. This large glycoconjugate is found on putative progenitor cells in the liver and the pancreas, and in intestinal metaplasia of the esophagus, GCTM-5 marks cells that appear to be progenitors in the development of metaplasia. GCTM-5pos cells isolated from adult liver were successfully expanded and passaged *in vitro*. GCTM-5 expression is retained through multiple passages and subsets of cells co-express liver progenitor markers N-CAM and EpCAM as well as hepatocyte (albumin) and biliary markers (CK19), suggesting a liver progenitor phenotype. By qPCR, cultured GCTM-5pos cells co-express albumin, 1-anti-trypsin, Hnf1, and Hnf6. In normal neonatal liver, GCTM-5 expression is restricted to a subset of biliary epithelial cells and cells within the canal of Hering where hepatic progenitor cells are purported to reside. In contast, livers from patients with biliary atresia exhibit a marked increase in GCTM-5pos cells within the expanding ductular reactive cells. Among the GCTM-5pos population are distinct groups of cells that are CK19pos, N-CAMpos, and EpCAMpos. GCTM-5pos cells also co-stain with Ki-67. Some GCTM-5pos cells are also CK19pos and albuminpos, suggesting a bipotential progenitor phenotype. The GCTM-5 antigen is a useful marker in studies of embryonic stem cell differentiation and renewal, repair, and regeneration in tissues of endodermal origin.

LUNG CELLS

Poster Board Number: 1050

THERAPEUTIC POTENTIAL OF HUMAN CORD BLOOD-DERIVED ENDOTHELIAL COLONY FORMING CELLS IN NEONATAL OXYGEN-INDUCED LUNG INJURY

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INTRODUCTION: Bronchopulmonary dysplasia (BPD) and emphysema are life-threatening lung diseases characterized by either impaired alveolar development or alveolar destruction. Both conditions lack effective therapy. There is growing evidence that angiogenic growth factors actively support alveolar growth and maintenance throughout postnatal life. However, the likely role of vascular progenitor cells (endothelial progenitor cells, EPCs) in lung growth and maintenance is unknown. OBJECTIVES: In this study, we examine the role of endothelial colony forming cells (ECFCs), an EPC subtype capable of self-renewal and *de novo* vessel formation, in lung de-

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velopment and repair. We hypothesize that ECFC-driven angiogenesis contributes to normal lung alveolar growth and ECFC supplementation restores disrupted alveolar development in hyperoxia-induced BPD. METHODS: *In vitro*, we assessed the proliferative, clonogenic and vessel forming potentials of lung ECFCs isolated from newborn rats housed in 95% O2 (BPD model) vs. room air controls. *In vivo*, the therapeutic potential of human umbilical cord blood (hUCB) ECFCs was assessed in immunocompromised Rag-/- mice exposed to 85% O2 from Postnatal day-(P) 6 to 14 and sacrificed at P-28. RESULTS: ECFCs isolated from hyperoxic lungs proliferated less rapidly when cultured *in vitro* for 3 weeks. More strikingly, ECFCs from the hyperoxic group showed decreased clonogenic capacity and formed fewer endothelial networks in matrigel. Intra-jugular administration of hUCB-ECFCs after established lung injury restored lung function and alveolar structure in hyperoxia-exposed mouse pups. ECFC therapy also attenuated pulmonary hypertension, a co-morbidity commonly associated with severe BPD. CONCLUSION: Impaired ECFC function in hyperoxia-exposed rat pups contributes to arrested alveolar growth in BPD. hUCB-derived ECFC may offer a new therapeutic avenue for lung diseases characterized by alveolar injury.

Poster Board Number: 1052

DIRECTED DIFFERENTIATION OF MOUSE ES CELLS INTO PRIMORDIAL NKX2.1+ LUNG AND THYROID PROGENITOR CELLS

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RATIONALE: During normal embryological development, a small population of foregut endoderm cells are lineage specified into lung progenitors that then give rise to the entire post-natal adult lung epithelium. The transcription factor Nkx2.1 is the earliest marker of these progenitors at gestational day ~E9 of mouse development. Nkx2.1 expression is also seen in developing thyroid and forebrain. Little is known about the identity and gene programs involved in lineage specification and differentiation of the lung progenitor cells. We aimed to recapitulate normal early anterior foregut endoderm development *in vitro* and purify primordial lung and thyroid progenitors, identified by Nkx2.1 expression, to better understand lung/thyroid development. METHODS: We developed a reporter ES cell line by targeting a cDNA encoding the GFP reporter gene into the Nkx2.1 locus by homologous recombination. These ESCs were differentiated efficiently to definitive endoderm using Acivin A-supplemented serum-free media. To attempt to recapitulate anterior and ventral endodermal patterning we used a combination of growth factors and TGFb/BMP inhibitors, resulting in the emergence of GFP-positive cells after 9 days in culture. GFP-positive cells were sorted by FACS and either analyzed by quantitative real-time PCR or re-plated and cultured in various media followed by qRT PCR for markers of lung epithelial cells. RESULTS: The reporter line was faithful and specific based on qRT-PCR evidence of endogenous Ttf1 mRNA expression solely in the FACS purified positive GFP fraction. Fluorescence microscopy revealed clusters of GFP+ cells of varying morphology, including epithelial-like structures from day 9 onwards. These cells expressed the endodermal marker Foxa2 and did not express neurectodermal markers above baseline undifferentiated stem cells. With further culture expansion and FGF2+10 growth factor stimulation, purified TTF1-GFP+ cells were lung and thyroid competent as evidenced by upregulation of markers SPC, SPB, CC10, FoxJ1, & CFTR suggesting a lung-like phenotype, and Thyroglobulin, Pax8 & TSHR suggesting a thyroid-like phenotype. CONCLUSIONS: Our in-vitro system yields a purified population of Ttf-1 positive endodermal cells of a molecular phenotype and differentiation repertoire reminiscent of primordial lung and thyroid progenitors.

Poster Board Number: 1054

RECRUITMENT OF p63-EXPRESSING DEEP AIRWAY STEM CELLS FOR LUNG REPAIR FOLLOWING H1N1 INFLUENZA INFECTION IN MICE

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The extent of lung repair following catastrophic damage and the potential role of adult stem cells in such processes remains obscure. Here we employ a murine-adapted H1N1 influenza virus related to that of the 1918 pandemic to introduce widespread but survivable damage to all regions of the mouse airways. We find that p63-expressing stem cells in the bronchiolar epithelium undergo massive proliferation after infection followed by a concerted radiation to interbronchiolar regions of alveolar ablation. Once there, these cells proliferate into discrete, Krt5+ pods and initiate the expression of markers typical of type 1 pneumocytes. Gene expression profiles of lung containing these pods suggest that they participate in a process of reconstituting the alveolar-capillary network eradicated by viral infection. The dynamics of this p63-expressing stem cell in lung repair mirrors recent findings that pedigrees of human deep airway stem cells assemble alveoli-like structures *in vitro* and suggests new therapeutic avenues to acute and chronic airway disease.

Poster Board Number: 1056

MODULATION OF K-RASG12V TRANSFORMATION BY P38ALPHA IN MOUSE LUNG PROGENITOR/STEM CELLS AND THEIR ROLE IN LUNG TUMOUR DEVELOPMENT

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Bronchioalveolar stem cells are expanded in the premalignant lesions of K-Ras-induced lung tumorigenesis. For this reason, bronchioalveolar stem cells have been proposed as the likely cell of origin in lung adenocarcinoma. We have previously shown that p38alpha attenuates K-Ras-induced lung cancer and transformation of bronchioalveolar stem cells; in absence of p38alpha, mice expressing the K-Ras oncogene show earlier initiation and progression to malignant stages of lung cancer. To define the mechanisms mediating p38alpha repression of lung tumorigenesis, we have isolated putative lung stem cells with the signature SPC+/CC10+/Sca1+/CD31-/CD34-/CD45-. These lung cells have been infected with retroviral constructs carrying a wild-type (WT) or oncogenic form of K-Ras. The oncogenic form of K-Ras (K-RasG12) is the most common K-Ras mutation found in both human and mouse lung adenocarcinoma. In addition these cells have been also infected to express a dominant negative mutant that blocks the p38alpha pathway. These cells maintain the profile signature of the wild-type lung stem cells. From a functional point of view, the K-RasG12V cells grow faster than the K-RasWT cells due to an accelerated G1-S phase transition induced by K-

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RasG12V. This increased proliferation is potentiated in absence of p38alpha activity. *In vitro* transformation of lung stem cells expressing K-RasG12V has been tested for their capacity to form more and bigger colonies in soft agar. This cell anchorage-independent proliferation is increased under deficient p38alpha pathway. We are also assessing the role of differentiation on K-RasG12V transformation and how K-Ras expression may influence the capacity of the lung stem cells to differentiate. Our studies are complemented with an *in vivo* model of K-RasG12D-induced lung tumorigenesis. We are using a TetOn system with a lung specific Sftpc-Tg activator that induces Cre activation after Doxycycline administration activating the K-RasG12D oncogene while deleting p38alpha-lox alleles. We are studying the specific role of cells with active Sftpc promoter (AT2 and lung stem cells) in the initiation and progression of lung cancer and the mechanisms involved.

Poster Board Number: 1058

HUMAN FETAL MESENCHYMAL STEM CELLS FOR THE TREATMENT OF A MURINE MODEL OF SMOKE-INDUCED ACUTE LUNG INJURY

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Introduction: Acute lung injury (ALI) can be complicated with the development of acute respiratory distress syndrome (ARDS), brought about by aggressive inflammatory responses in the alveolar system. Current treatment for ALI/ARDS is empiric with mortality rates of up to 40% despite advances with supportive treatment, necessitating alternative treatment modalities. There is mounting evidence that MSC may be able to alleviate this inflammatory response in different models of lung injury. Here we develop a murine model of smoke-induced ALI and investigated the ability of fetal MSC (fMSC) to ameliorate the lung injury. Methods: fMSC were isolated from fetal bone marrow and characterised. Global gene expression of fMSC was studied using microarray studies, where fMSC were first subjected to TNF-a stimulation to represent tissue injury. fMSC and endothelial cells co-culture systems were employed to study effects on vascular formation and stabilisation. Finally, a murine model of ALI via smoke inhalation was developed. Results: TNF-a stimulation of fMSC resulted in 170 genes differentially regulated. In particular, inflammatory pathway genes like C1 inhibitor (5-fold), IL-28RA (4-fold), phospholipase A2, group X and prostacyclin synthase (3-fold) were up regulated, where as chemokine (C-C motif) ligand 2, IL-8 and IL-9R were down regulated more than two-fold. These results highlight the immunomodulatory response of fMSCs *in vitro* that could facilitate the amelioration of lung injury. Endothelial cells seeded on to fMSC monolayers exhibit network structures implicating the potential for tissue repair following vascular permeability in an injured lung. NOD/SCID mice exposed to smoke inhalation results in acute lung injury due to severe inflammatory response shown by the presence of neutrophils in histological lung sections. The effect of fMSC on this ALI model will be discussed. Conclusion: A murine model of ALI due to smoke inhalation has been developed. Together with the *in vitro* studies that demonstrate the immunomodulatory and tissue regenerative properties of fMSC, there is much potential for fMSCs to be used as treatment for ALI.

Poster Board Number: 1060

POLYETHYLENEIMINE (PEI)-MEDIATED TRANSIENT AND STABLE *IN VIVO* GENE DELIVERY IN LUNG PROGENITOR CELLS, AND ITS APPLICATIONS FOR ACUTE LUNG INJURY (ALI) TREATMENT AND TUMOR GROWTH INHIBITION

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Polyethyleneimine (PEI) is a cationic polymer that is effective for DNA complex and gene transfer. Systemic administration of PEI/DNA complex in mice mediates gene delivery in lung. The gene expression is efficient and can be detected in 6 hours post-delivery, but then it reduces and vanishes rapidly in the following days. Transgene expression is localized to the alveolar region with the 2 types of alveolar epithelial cells (AECs) as major target. Plasmid DNA incorporating a transposon system, such as Sleeping-Beauty (SB) or piggyBac (PB), can address the problem of transient duration and induce a long-term transgene expression in mouse lung after PEI-mediated delivery. We have found that the stabilization of transposon-conveyed gene expression in lung actually arose from the multiplication of a small number of transposon-integrated alveolar progenitor cells, which proliferated both under normal conditions and in response to local injury for epithelia repair, and may play a role in long-term homeostatic maintenance in alveoli. In this report, we show that the transposon-labeled progenitor cells can be isolated and cultured *ex vivo*. In addition, the PEI-mediated transient gene delivery is applicable to acute lung injury (ALI) treatment. Following the ALI induced by intratracheal instillation of lipopolysaccharide (LPS), systemic delivery of PEI/DNA expressing β2-adrenergic receptor (β2AR), a prototypical G protein-coupled receptor that regulates several key proteins needed for alveolar epithelial ion and fluid transport, efficiently reduced the lung wet-to-dry ratio and bronchoalveolar lavage protein levels and cellularity in 24 hours without remarkable inflammation or other adverse effects. On the other hand, the stable delivery of tumor suppressor genes, such as p27VP22 and soluble FLT-1, in alveolar progenitor cells, significantly inhibited the local tumor growth through bystander effect and prolonged the survival of treated mice. The data presented here suggest that PEI-mediated *in vivo* gene delivery in lung progenitor cells can serve as a versatile tool for treatment of acute and chronic lung diseases, and may also provide a promising platform for future research in lung biology and tissue regeneration.

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CARDIAC CELLS

Poster Board Number: 1062

HUMAN CARDIAC STEM CELLS – THERE IS A POOL OF AUTOLOGOUS RESIDENT C-KIT+ PROGENITORS IN THE LEFT AND RIGHT ATRIUM

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Objectives: The existence of resident human cardiac stem cells (hCSC) in the adult myocardium and their regenerative capacity is not fully defined. The aim of this study was to identify hCSC in patients undergoing heart surgery by Flow Cytometry. Furthermore we wanted to investigate if there are differences in the distribution of these cells in different compartments of the human heart. Methods: Tissue samples from the appendage of the right atrium (RA), left atrium (LA) and from the left ventricle (LV) were taken during cardiac surgery. The samples were digested and the mononuclear cells were isolated and marked for the stem cell marker c-kit and hematopoietic lineage markers (CD3, CD11b, CD19 and CD45). Quantitative and qualitative cell characteristics were analyzed by Flow Cytometry. Furthermore immunohistology was performed. Results: Human cardiac stem cells, positive for c-kit and negative for hematopoietic lineage markers could be isolated from the RA, LA and LV. There is no significant quantitative difference of c-kitpos/linageneg cells (RA 4.80±1.76% vs. LA 4.99±1.69% of isolated MNCs, P=0.922) between the atria. There is a significant difference of c-kitpos/linageneg cells between the atria and the left ventricle (atria 4.90±1.29% vs. LV 0.62±0.14% of isolated MNCs P=0.035). The immunohistology showed c-kitpos/linneg cells in all compartments and confirmed the distributional pattern shown by Flow Cytometry. Conclusion: We could detect c-kitpos/linageneg cell populations in the heart of patients undergoing cardiac surgery. There is no difference in the distribution between RV and LV, but we showed a higher concentration in both atria compared to the LV.
Explanting tissue samples from the atria and isolating, promoting and transplanting the hCSCs might be an endogenous source for regeneration of heart failure.

Poster Board Number: 1064

A UNIVERSAL SYSTEM FOR HIGHLY EFFICIENT CARDIAC DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS THAT ELIMINATES INTERLINE VARIABILITY

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The production of cardiomyocytes from human induced pluripotent stem cells (hiPSC) holds great promise for patient-specific cardiotoxicity drug testing, disease modeling, and cardiac regeneration. However, existing protocols for the differentiation of hiPSC to the cardiac lineage are inefficient and highly variable. We describe a highly efficient system for differentiation of human embryonic stem cells (hESC) and hiPSC to the cardiac lineage. This system eliminated the variability in cardiac differentiation capacity of a variety of human pluripotent stem cells (hPSC), including hiPSC generated using non-viral, non-integrating methods. We systematically and rigorously optimized >45 experimental variables to develop a universal cardiac differentiation system that produced contracting human embryoid bodies (hEB) with an improved efficiency of 94.7±2.4% in an accelerated nine days from four hESC and seven hiPSC lines tested, including hiPSC derived from neonatal CD34+ cord blood cells and adult fibroblasts using non-integrating episomal plasmids. This cost-effective differentiation method employed forced aggregation hEB formation in a chemically defined medium, along with staged exposure to physiological (5%) oxygen, and optimized concentrations of mesodermal morphogens BMP4 and FGF2, polyvinyl alcohol, serum, and insulin. The contracting hEB derived using these methods were composed of high percentages (64-89%) of cardiac troponin I+ cells that displayed ultrastructural properties of functional cardiomyocytes and uniform electrophysiological profiles responsive to cardioactive drugs. This efficient and cost effective, universal system for cardiac differentiation of hiPSC allows the potentially unlimited production of functional cardiomyocytes suitable for application to hPSC-based drug development, cardiac disease modeling, and the future generation of clinically-safe human cardiac cells for regenerative medicine.

Poster Board Number: 1066

TRANSDIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS INTO CARDIOMYOCYTES USING MYOCARDIUM TISSUE OBTAINED FROM DECEASED PATIENTS

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The increase of life expectancy has led to a high percentage of aging population in the developed world and consequently, the high incidence of degenerative and cardiovascular diseases. Regenerative medicine is presented as a promising tool for the treatment of cardiovascular diseases through the cardiac regeneration. The ventricular remodelling is the natural process of resolution of Acute Myocardial Infarction (AMI), which causes apoptosis and necrosis with scar formation, progressive ventricular dilatation and heart failure ending in death. A current therapeutic strategy is to increase the number of human functional heart muscle through the transplantation of several adult stem cell types. The use of human Mesenchymal Stem Cells (hMSCs) represents an attractive alternative because of their capacity for proliferation, easy collection and its immunological and paracrine properties, which could include the activation of different mechanisms against cell damage. They are multipotent cells that are present in many adult tissues, including bone marrow and adipose, etc., that in response to specific culture conditions, these cells can give rise to multiple mesenchyme-derived cell types. This type of cross-lineage differentiation, also known as transdifferentiation, is a process whereby one cell type committed to and progressing along a specific developmental lineage switches into another cell type of a different lineage through genetic reprogramming. This phenomenon implies that adult stem cells maintain the multidifferentiation potentials even after being exposed to certain inductive factors. The aim of this study was to develop an experimental model of cardiac regeneration consisting in obtain an optimum number of transdifferentiated hMSCs into cardiomyocytes, using techniques such as cell extract, co-culture and conditioned medium. For this purpose, we used hMSCs obtained from lipoaspirates and cardiomyocytes isolated from left ventricular samples of deceased patients (with dates of death less than 48 hours). We used several strategies to induce cardiac differentiation consisting in: (i) a protein pool developed and introduced into hMSCs through membrane cell permebilization (cell extract method), (ii) a co-

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culture method using transwells with both cell types and (iii) the addition of conditioned medium collected from the adult cardiomyocytes isolated to the hMSCs (conditioned medium method). After two weeks of culture for each method, morphological studies were conducted and genetic determinations by RT-PCR were performed. We evaluated the expression of cardiomyocyte differentiation factors: GATA-4, Mef2c and Troponin C, induction factors such as BMP-2. Moreover, immunocytochemistry assays were performed for the expression of proteins such as desmin, α-actin, troponin I and C in the treated cells. Our results showed that hMSCs obtained from lipoaspirates can be reprogrammed into cardiomyocytes through the transdifferentiation methods tested. We conclude that heart tissue obtained from deceased patients can be a valuable source of cardiomyocytes for use in methods of cell reprogramming. This study elucidates the first insight that hMSCs with self renewal potential possess the capability to serve as a functional cardiomyocyte precursor through the appropriate paracrine input and cross-talk with the cardiac milieu.

Poster Board Number: 1068

IDENTIFICATION AND CHARACTERIZATION OF NOVEL AND SPECIFIC MARKERS FOR CELLS OF THE HUMAN CARDIOVASCULAR LINEAGE

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The potential of embryonic stem (ES) cells and induced pluripotent stem (iPS) cells to generate cells of the cardiovascular lineage in culture provides a powerful model system for investigating the cellular interactions and molecular regulators that govern the specification, commitment and maturation of these lineages. Studies in mouse and human have identified a Flk-1/KDR-positive cardiovascular progenitor that gives rise to cells of the cardiac, vascular smooth muscle and endothelial cell lineages. When plated in monolayer cultures, these Flk-1/KDR+ cells can differentiate to generate populations consisting of greater than 50% contracting cardiomyocytes. While the mechanisms regulating the early differentiation steps of the cardiovascular lineage are rather well defined and relatively easy to monitor, the pathways that control the differentiation and maturation of the progenitor population into the distinct cell types of the adult heart (atrial and ventricular myocytes, cardiac conduction system, heart valves) are largely unknown. One major limitation in defining and characterizing the pathways that regulate fate choice and cell maturation within the cardiovascular lineage is the paucity of markers to monitor the emergence of the individual cell types and to enable their isolation from mixed cultures. Various studies have reported cell type specific reporters or purification based on mitochondrial dye up-take, however, none of these approaches have proven to be applicable to a broad range of human ES and iPS cells or to be efficient in consistently obtaining highly enriched cardiomyocyte populations. Using high throughput flow cytometric analysis we have identified a novel cell surface marker that is specifically expressed on hESC/iPSC-derived and fetal cardiomyocytes. Cell sorting using an antibody against this marker allows for enrichment of cardiomyocytes from human ESC/iPSCPS differentiation cultures, resulting in populations consisting of greater than 95% cardiac TroponinT-positive cells. When subjected to gene expression analysis, these sorted cells express high levels of cardiac-specific markers such as NKX2.5, MYH6, MYH7, MYL2, MYL7 and are devoid of markers characteristic for the non-myocyte cell types of the cardiovascular lineage including smooth muscle cells and fibroblasts. The non-myocyte markers are, however, expressed in the antibody-negative fraction. When plated in culture, the sorted cells give rise to contracting monolayers or aggregates that maintain their high cardiomyocyte content over time. CONCLUSION: We have successfully identified a novel marker of developing cardiac lineage cells that can be used to isolate cardiomyocytes from hESC/iPSC-derived populations with high efficiency and specificity. This novel and widely applicable tool will be invaluable for the generation of pure cardiomyocyte populations for future strategies in regenerative medicine as well as drug testing approaches for human PSC-derived cardiomyocytes. Furthermore, being able to monitor and isolate cardiomyocytes during PSC differentiation will open new possibilities to understand and characterize the pathways and molecular regulators involved in the formation of the human heart in healthy and diseased patients.

Poster Board Number: 1070

AUTOMATED FUNCTIONAL CELLULAR ANALYSES OF HUMAN IPS-DERIVED CARDIOMYOCYTES FOR CARDIOVASCULAR RESEARCH, TOXICITY AND DRUG DISCOVERY

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Human induced pluripotent stem cells (iPSC) are an ideal source of functionally relevant, terminally differentiated cells for use in research, drug discovery, and toxicity testing. They are available as pure populations that can be supplied in the quantities required for drug discovery and screening campaigns. iPSC-derived cardiomyocytes are attractive because they express ion channels and demonstrate beating and action potentials similar to primary cardiac cells. Human cardiomyocytes derived from stem cell sources can greatly accelerate the discovery of cardiac drugs and safety pharmacology by offering more clinically relevant human culture models than presently available. An emerging application for iPS-derived cardiomyocytes is for use as a model human system for testing toxicity and cardioprotective effects of drug candidates and developing drugs. Here we described methods that can be used to simultaneously monitor cell viability and mitochondria integrity in an automated fashion using high-content imaging. We have used iPS-derived cardiomyocytes to determine IC50s of several cardiotoxic compounds (Doxazosin, Imatinib, Antimycin A, Terfenadine, Verapamil). A combination of cell scoring and granularity algorithms was used for analysis of the impact of compounds at the sub-cellular level. This method also evaluates the cardioprotective effect of other compounds (sphingosine phosphate) against cytotoxic damage. Since iPSC-derived cardiomyocytes demonstrate spontaneous beating similar to primary cardiac cells, we demonstrate an assay for measuring the impact of pharmacological compounds on the rate and magnitude of cells beating in 96 or 384 well microplates. We have developed a protocol that enables automatic determination of beat rate and magnitude of iPS-derived cardiomyocytes from time-lapse images of live cardiomyocytes, allowing measurement of the effect of compounds such as epinephrine and caffeine. In addition, iPSC-derived cardiomyocytes have been evaluated for their potential in ion channel drug discovery. Preliminary studies have demonstrated functional K and Ca channels using an automated patch clamp instrument which could then be used for screening prospective drugs.

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SECONDARY SPHERE FORMATION ENHANCES THE FUNCTIONALITY OF CARDIAC PROGENITOR CELLS FROM MURINE AND HUMAN CARDIAC EXPLANTS

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Objective: We investigated a new strategy that enhances cellular potency and provides an enriched source of cardiac progenitor cells. Background: Recent studies suggest that resident cardiac stem/progenitor cells could be an optimal cell source for repairing a damaged heart. However, cardiac progenitor cells are difficult to maintain in terms of purity and multipotency when propagated in attached 2-dimensional culture systems *in vitro*. Methods: We applied the repeated sphere formation strategy (that is, cardiac explant ; migrating cells out of explant ; primary cardiosphere (CS) formation ; cardiosphere-derived cells (CDCs) in adherent culture condition ; secondary CS formation by 3-dimensional culture). Results: Murine CSs were generated from cardiac explants of C57BL/6 mice. Cells in secondary CS showed a higher potency and differentiation potentials. Furthermore, when transplanted into the infarcted myocardium, secondary CSs engrafted robustly, improved LV dysfunction, and reduced infarct sizes more than CDCs did. In addition to the cardiovascular differentiation of transplanted secondary CSs, paracrine effects, such as robust VEGF secretion, were found to enhance neovascularization in the infarcted myocardium. Regarding the molecular mechanisms involved, ERK/E-selectin and ERK/Sp1/VEGF auto-paracrine loop were responsible for sphere initiation and maturation, respectively. When we applied this strategy to adult human cardiac tissues, secondary CS was readily generated, and cellular characteristics and molecular pathways of sphere formation were similar to murine findings. Conclusion: These results provide a simple and reproducible strategy for enhancing cellular potency for cardiac repair. Furthermore, this strategy may be applicable to other types of stem/progenitor cells for cell-based therapy.

Poster Board Number: 1074

ANTHRACYCLINE INDUCED CELLULAR DAMAGE DURING HUMAN PLURIPOTENT STEM CELL CARDIAC DIFFERENTIATION

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Backround: Anthracycline (ANTs) mediated cardiotoxicity is a leading cause of late complications in cancer survivors. The mechanisms of cardiotoxicity of ANTs have been studied *in vivo* and *in vitro* in animal models. We investigated the role of oxidative stress and DNA damage in mediating ANT-induced cardiotoxicity using human pluripotent stem cell-cardiac cells (hPSC-CCs). Methods: The hPSC-CCs were treated at stage T15 with doxorubicin (DOXO, 0.1, 0.5, and 1 μM/L) with or without the antioxidant, ascorbic acid (AA, 200 μM/L) for 48hrs. The effect of DOXO on the following was measured: 1) 8-OH-deoxyguanosine (8-OH-dG), marker of reactive oxygen species (ROS) by Elisa assay; 2) Phosphorylation of p53 at ser6, as a marker of DNA damage by immunofluorescence staining, and 3) beating rate of contracting embryonic bodies (EBs). Results: DOXO treatment of hPSC-CCs for 48 hours resulted in: 1) increase in 8-OH-dG levels which was attenuated by co-treatment with ascorbic acid (9.3±2.1 and 4.3±3.3 ng/ml in DOXO and DOXO+AA groups compared to control 3.8±2.3 ng/ml, $p<0.05$) 2) increase in phospho-p53 at ser6 in DOXO group compared to control; and 3) a dose-dependent reduction in contractile rate of EBs (40 ± 2.1% in 0.1, 12±1.4% in 0.5, and 1.3±1.1% in 1 μM/L concentration exposure of DOXO, vs 96±2.4 % control $p<0.05$). This was not altered by AA. Conclusion: ANT administration induced significant increase in ROS, DNA damage and contractile dysfunction in hPSC-CCs. Antioxidant administration reduced the ROS levels but did not prevent DNA damage or contractile dysfunction. The hPSC-CCs may serve as a useful *in vitro* cellular model to study the mechanisms of ANT-mediated cardiotoxicity and screen for newer cardioprotective agents.

Poster Board Number: 1076

DEVELOPMENT OF DELAYED AFTERDEPOLARIZATIONS ACTIVITY IN HUMAN IPS CARDIOMYOCYTES

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Background: Human induced pluripotent stem cell (hiPS)-derived cardiomyocytes exhibit diverse electrophysiological and pharmacological properties. One of the goals of hiPS cardiomyocyte development is to have available a human model suitable for safety pharmacology. The present study tests the hypothesis that agents known to induce delayed (DAD) afterdepolarizations do so in hiPS cardiomyocytes. Method: Embryoid bodies (EBs) were made from a human iPS cell line reprogrammed with Oct4, Nanog, Lin28 and Sox2 using a serum free differentiation protocol supplemented with growth factors for selective cardiac differentiation. Beating clusters were micro-dissected from EBs ranging between 70 and 80 days of maturity and plated on gelatin coated dishes. Standard microelectrode techniques were used to record action potentials (AP) from electrically stimulated EB clusters superfused with HEPES-Tyrode's solution (KCl=4 mM; 37°C) before and after exposure to 2.5-5.0 μM ouabain (n= 6). Results: The effects of ouabain were examined using a pulse train of 10 beats at cycle lengths (CLs) of 1000, 800, 500 and 300 ms followed by a pause. Ouabain (2.5-5 μM) reduced phase 4 depolarization and induced DADs in 3/6 and DADs-induced triggered activity in 1/6 preparations. DAD amplitude increased progressively as CL was reduced, consistent with the characteristics of DADs observed in native cardiomyocytes. Conclusion: Our data indicate that cardiomyocyte clusters derived from hiPS generate DAD-induced triggered activity consistent with their toxicological effects in the clinic.

Poster Board Number: 1078

QUICK INDUCTION OF FUNCTIONAL MATURATION IN CARDIOMYOCYTES DERIVED FROM HUMAN ES AND IPS CELLS

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Cardiomyocytes derived from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are considered a promising cell source for repairing cardiac dysfunction or for drug discovery and development. Most recent research has focused on efficient cardiac-cell induction procedures which convert pluripotent stem cells into contractile cardiomyocytes. Unfortunately, the resulting cells are often functionally insufficient

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as adult cardiomyocytes and generally possess properties more similar to those of fetal cardiomyocytes. In order to reproduce the developmental process seen in hearts during culturing of hESC/hiPSC-derived cardiac cells, additional steps designed to promote their full maturity need to be taken. We previously showed that the combination of short-term spheroid culturing following periodic re-plating of hESC-derived cardiomyocyte colonies produced cells which maintained their contractility for over a year, resulting in a cell-autonomous functional increase in cardiac gene expression and electrophysiological properties. Here, we found that spheroid culturing activated the chromatin structure of hESC-derived cardiomyocytes. Moreover, direct modification of the chromatin structure with a specific compound enhanced the cardiac gene expression in the premature cardiac cells obtained from both hES and hiPS cells within a few days under the adherent culture conditions. In the extracellular multielectrode recordings, premature cardiac colonies often exhibited arrhythmia, but with this treatment, most of them showed dose-dependent Na+-K+ interval prolongation in response to a HERG channel blocker. This suggests that proper chromatin regulation is essential for the stem cell-derived cardiomyocytes to acquire full functionality. Our one-step pretreatment of a cardiomyocyte colony within each electrode chamber will greatly contribute to the accuracy of cell toxicity tests on the basis of the Na+-K+ interval prolongation in the drug discovery process.

Poster Board Number: 1080

APPLICATION OF HUMAN EMBRYONIC DERIVED CARDIOMYOCYTES IN TOXICITY TESTING

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The lack of readily available human cardiac tissue has hindered progress in safety pharmacology and drug discovery. Human embryonic stem cell (hESC) derived cardiomyocytes are a potential source of relevant cells to substitute for *ex vivo* material. These cardiomyocytes have been characterised at the genetic, molecular and functional level, and demonstrate typical cardiac characteristics. We now address the application of these cells in assessing new chemical entities for cardiotoxicity. hESC derived cardiomyocytes were seeded as single cells, or maintained as 3D structures, and were exposed to compounds with documented adverse cardiac events. Toxicity was measured by several parameters, including cell viability and release of the cardiac specific biomarker cardiac troponin t (cTnT). Doxorubicin, an anthracycline, is one of the most widely used antitumor drugs with several potential mechanisms underlying the cardiotoxic effects. Doxorubicin caused a dose dependent decrease in viability and increase in cTnT release. Interestingly, these effects were also time-dependent, and sensitivity was affected by proliferative capacity. Metformin, a commonly prescribed drug for use by type II diabetics, had dual effects, with no decrease in cell death at low doses but reduced cell viability only at high concentrations. This pattern was closely reflected by the release of cTnT. Toxicity testing was performed using serum free and serum containing media, and in 96- or 384-well plates, to assess transferability to high throughput systems. The viability of hESC derived cardiomyocytes was not affected by the absence of serum during the test period, nor by the constrained environment of the 384-well plate. In summary, hESC derived cardiomyocytes responded to cardiotoxic compounds with a sensitivity comparable to that reported in literature in formats suitable for large scale screening campaigns. hESC derived cardiomyocytes have a potential to provide human cardiac material for toxicity testing. Frontloading of hESC derived cardiomyocytes in screening new chemical entities for adverse cardio events could provide more relevant information during the early stages of compound development.

Poster Board Number: 1082

ILK INDUCES STRESS IN MOUSE HEART AND PROMOTES EXPANSION OF RESIDENT CARDIAC STEM CELLS

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Background: We have previously reported that Integrin Linked Kinase (ILK), which has been implicated as a cardioprotective target, causes the *in vitro* expansion of cardiac c-kit + tissue-resident progenitor cells in human fetal heart. However, the effect of ILK on expansion of other cardiac progenitor cells remains obscure. Methods and Results: To investigate the role of ILK in expansion of resident stem cells we used transgenic mice with cardiac restricted expression of constitutively active ILK (S343D) or activation resistant mutant ILK (ILK-R211A). We isolated sca-1+ cells from hearts of ILK-S343D and ILK R211A transgenic mice using magnetic cell sorting technique. The percentage of Sca1+ cardiac progenitor cells was significantly increased in both ILK over-expressed hearts of transgenic mice, with the highest increase (3-4 fold) in R211A mice as compared that of littermate control mice. RT-PCR analysis of Sca 1- cells isolated from hearts of ILK S343 and ILK R211A mice expressed markedly higher levels of cardiac progenitor cell-marker Isl-1, as compared to expression levels of Isl-1 in Sca-1- cells isolated from hearts of littermate controls. Moreover, transgenic mice with cardiac-specific ILK overexpression were shown to have increased myocardial Isl-1 protein expression level with the highest expression levels in R211A mice. Microarray analysis revealed induction of a robust heat-shock response. Furthermore, ILK immunoprecipitation show specific binding of ILK with constitutively active heat shock protein 70 (Hsc-70) in both ILK wild type and ILK mutants over-expressed in the hearts of transgenic mice. Conclusion: Cardiac-specific upregulation of ILK expression induces stress conditions in mouse heart with specific binding of ILK with Hsc-70, and promotes expansion of various resident cardiac stem cells, such as c-kit+-, Sca1+- and Isl-1+ -expressing cells.

Poster Board Number: 1084

LACK OF POLYCOMB PROTEIN RYBP/DEDAF IN MOUSE EMBRYONIC STEM CELLS IMPAIRS CARDIAC DIFFERENTIATION

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Polycomb protein Rybp/DEDAF (Rybp (Ring1 and YY1-Binding Protein; also known as DEDAF; UniGene Mm.321633; MGI:1929059) has been implicated in transcriptional regulation, apoptotic signaling and as a gene with important function in central nervous system development. Earlier, we have proven that RYBP plays important role in neural tube formation and development of the eye. However the precise molecular functions of Rybp/DEDAF and its role during organogenesis remained still unclear. In present study, we investigated the role of RYBP during heart development. To gain further support for the early embryonic expression pattern of Rybp in the embryonic heart, we determined the Rybp protein expression profile throughout heart development by utilizing anti-Rybp antibody on embryonic sections. Rybp was shown to be readily detectable in the developing heart from day 7.5 of embryonic development (E7.5). Prominent Rybp expression persisted during all embryonic stages examined and RYBP marked differentiated cell types of the heart suggesting its involvement in heart development. This prompted us to investigate the role of RYBP in the regulation of cardiac lineage commitment in a stem-cell model system. In order to investigate the function of RYBP during cardiac differentiation, we utilized RYBP null embry-

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onic stem cells (ESCs) and differentiated them towards cardiac lineages. We used the hanging drop method to generate embryoid bodies (EBs) as common intermediate during the *in vitro* differentiation of pluripotent stem ESCs into specialized cell types. We found although EBs could be derived from the RYBP null ESCs but consequent cardiac development was affected in the absence of RYBP. Furthermore, normally ESCs form rhythmically beating cardiomyocytes in 6-14 days when EBs plated on gelatin coated surfaces. However no beating colonies were observed from the mutant cells. Analysis of cardiac markers revealed a down-regulation of terminal cardiac markers (e.g. cardiac troponinT (cTnT) in the mutants, assessed by immunohistochemistry and qRT-PCR. Importantly, forced expression of Rybp (by a lentiviral vector) in Rybp-deficient ESCs was able to rescue the mutant phenotype. In summary, this is a first attempt to understand the basis for Rybp's integral role in the development of the heart. Our results showed that Rybp is expressed in the heart during mouse embryonic development and that in the absence of RYBP cells show defect in cardiac differentiation. Currently gene expression profile analysis is being performed to reveal the genetic network RYBP may affect. These findings are likely to place RYBP in processes of cardiac differentiation and have implications for the understanding of heart diseases.

Poster Board Number: 1086

IDENTIFICATION OF GENES THAT REGULATE CARDIOMYOGENESIS IN MOUSE PLURIPOTENT EMBRYONIC CELLS

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The application of pluriopotent stem cells to cardiac repair holds great promise for stem cell-based therapy. An understanding of the mechanism underlying the cardiac differentiation of pluripotent stem cells is necessary for the effective generation and expansion of cardiomyocytes as cell therapy products. Here we have identified genes that modulate the cardiac differentiation of embryonal carcinoma (EC) cells and embryonic stem (ES) cells. We isolated P19CL6 cell sublines that possess distinct properties in cardiomyogenesis and extracted 24 cardiomyogenesis-related candidate (CMR) genes correlated with EC cell cardiomyogenesis by transcriptome analysis. Knockdown of the CMR genes by RNA interference (RNAi) revealed that 19 CMR genes influence spontaneous contraction or transcript levels of cardiac marker genes in P19CL6 cells. We further performed knockdown of the CMR genes by RNAi in mouse ES cells and induced *in vitro* cardiac differentiation. Three CMR genes were finally shown to modulate the cardiac differentiation of both EC cells and ES cells. We found that CMR2 is the most potent regulator of cardiomyogenesis in our identified CMR genes. Depletion of CMR2 attenuated the expression of early cardiac transcription factor Nkx2.5 without affecting transcript levels of pluripotency and early mesoderm marker genes during ES cell differentiation. Activation of Wnt/β-catenin signaling enhanced the expression of both CMR2 and Nkx2.5 in ES cells during embryoid body formation. Our findings indicate that CMR2 is a novel regulator of cardiomyogenesis in pluripotent embryonic cells, which links Wnt/β-catenin signaling to Nkx2.5 expression.

Poster Board Number: 1088

INVESTIGATING THE MECHANISM OF MURINE ATRIAL BI-POTENCY

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Within the developing heart, a subset of Isl1-postive cardiac progenitors display multi-potency and are capable of giving rise to cardiac, smooth muscle and endothelial cells. While these progenitors lose their ability to differentiate into endothelial cells early during heart development, the cardiac and smooth muscle lineages stay closely related until late cardiogenesis. One specific fraction of these progenitors is the cells of the posterior second heart field, which have already committed to the atrial lineage, but retain smooth muscle bi-potency until mid-gestation. The question of what molecular mechanism regulates the bi-potency of these cells and what developmental purposes this plasticity may serve remain largely unanswered. We hypothesize that that the cardiac-smooth muscle bi-potency of atrial progenitors is regulated by a Myocardin dependant pathway and is critical for the formation of functional heart-vessel junctions such as between the in-flow tract and atria of the heart. Using the atrial-specific sarcolipin (Sln)-Cre knock-in mouse line, in combination with an adenoviral myocardin over-expression system, we have shown that the over-expression of myocardin is capable of enhancing the smooth muscle differentiation of cultured atrial progenitor cells. Chimeric analysis shows that myocardin-null ES cells give little contribution to the smooth muscle layer within the cardiac inflow tract while preferentially contributing to the atrial myocardium, suggesting myocardin is required for the smooth muscle differentiation of atrial progenitors. Furthermore, we have begun to analyze this requirement *in vivo* using the Sln-Cre line to conditionally knock out the myocardin gene within the atrial chambers of the heart.

Poster Board Number: 1090

MHC6 ANTISENSE RNA EXPRESSION IN MURINE EMBRYONIC STEM CELLS LEADS TO LOSS OF THE CARDIOGENESIS PHENOTYPE

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Global transcriptomic analysis of murine embryonic stem cell derived cardiomyocytes led to the identification of Mhc6, the cellular role of which has not yet been reported till date despite of its higher abundance specifically in heart and brain. Functional analysis of this transcript in Zebrafish model with morpholino mediated knockdown showed severe abnormalities of the heart and blood vasculatures such as enlarged ventricles, atria and stagnancy of blood in blood vessels and eventually caused embryonic lethality with 100% percent. shRNA mediated constitutive knockdown of Mhc6 in embryonic stem cells and differentiating embryoid bodies showed complete abrogation cardiomyogenesis and null expression of canonical cardiac markers such as Troponin T and alpha-myosin heavy chain. Cellular localization studies indicated the nuclear/membrane localization of this protein and subsequent microarray analysis showed its functional role in mesoderm development and cardiomyogenesis. Here we report that the novel transcript Mhc6 is a critical component of cardiomyogenesis *in vivo* and *in vitro*.

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Poster Board Number: 1092

LONG QT SYNDROME SPECIFIC CARDIAC CELL MODELS GENERATED USING INDUCED PLURIPOTENT STEM CELL TECHNOLOGY

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Cell culture is an ideal environment to carry out biological and biomedical research but unfortunately, not all human cells can be maintained *in vitro*. Cardiomyocytes, for example, tend to dedifferentiate and stop beating relatively fast in cell culture conditions. Pluripotent human stem cells are capable to proliferate indefinitely or alternatively differentiate into any cell type of human body. Induced pluripotent stem (iPS) cells can be generated by reprogramming already differentiated cells into a pluripotent state by activating certain pluripotency genes. iPS cell technology thereby provides a way to study patient and disease specific cell lines. Here we introduce functional cell models for long QT syndrome (LQTS) type 1 (LQT1) and 2 (LQT2). LQTS can be seen in ECG of a patient as prolonged QT interval and the syndrome is known to increase the risk of ventricular arrhythmias and sudden death. The most common subtypes of the disease are due to mutations in KCNQ1 and HERG genes. Both of these genes code subunits of certain voltage-gated potassium channels that are responsible for re-polarization of cardiac action potential and maintenance of normal cardiac rhythm. The disease phenotype is also present in the LQTS-specific cardiomyocytes differentiated from iPS cells that were derived from skin fibroblasts of patients with LQTS. The phenotype is seen as prolonged cardiac action potential duration (APD) using patch clamp-technique as well as longer field potential duration (FPD) measured with micro electrode array (MEA). We have been able to generate cardiac cell models for both LQT1 (carrying mutation in KCNQ1) and LQT2 (carrying defective HERG). These human cardiac models for LQTS provide optimal platforms to study the basic pathology of the different subtypes of the syndrome and their responsiveness to different drugs. There are huge advantages compared to previous models that have used non-cardiac cells or animals since the intracellular environment is similar to real human heart. The models might also be used to safely design an optimal medication for the patients.

Poster Board Number: 1094

USING HUMAN INDUCED PLURIPOTENT STEM CELLS TO INVESTIGATE CARDIAC PHENOTYPES IN PATIENTS WITH TIMOTHY SYNDROME

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Prolonged QT interval, the electrical manifestation of repolarization in ventricular myocytes, is a major cause of cardiac arrhythmia and sudden death. Long QT syndrome (LQTS) can have a genetic basis or be induced by drug exposure or physiological stress. A missense mutation in the L-type calcium channel, Cav1.2, causes LQTS and arrhythmia in patients with Timothy syndrome. To examine how the TS mutation affects electrical activity and contractility in human cardiomyocytes, we developed a human model of Timothy syndrome by reprogramming human skin cells from TS patients to generate pluripotent stem cells and by differentiating these cells into cardiomyocytes. Using electrophysiological recordings and live cell imaging, we found that significant defects in rhythmicity, electrical activity and calcium signaling in Timothy syndrome cardiomyocytes, likely reflecting the cardiac phenotypes that are observed in the patients. To rescue the cellular phenotypes of Timothy syndrome patient-derived cardiomyocytes, we tested clinically-used blockers and chemical compounds that can alter the calcium channel function, and found that roscovitine, a compound that increases the voltage-dependent inactivation of Cav1.2, restored the electrical and calcium signaling properties of cardiomyocytes derived from Timothy syndrome. This study opens new avenues for studying molecular and cellular mechanisms of genetic cardiac arrhythmia in human and for developing new drugs to treat these diseases.

Poster Board Number: 1096

PARATHYROID HORMONE IS A DIRECT DPP-IV INHIBITOR AND THEREFORE INCREASES HOMING TO THE ISCHEMIC MYOCARDIUM VIA THE SDF-1/CXCR4-AXIS

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Aims: Parathyroid hormone (PTH) has been shown to induce stem cell proliferation in the bone marrow (BM) niche and to promote stem cell mobilization into peripheral blood. Moreover, PTH treatment after myocardial infarction improved survival and myocardial function associated with enhanced homing of bone marrow-derived stem cells (BMCs). To unravel the molecular mechanisms of PTH mediated stem cell trafficking, we analyzed wildtyp (wt) and GFP-transgenic mice after myocardial infarction with respect to the pivotal SDF-1/CXCR4 axis. Methods and Results: WT and GFP-transgenic mice (C57BL/6J) were infarcted by coronary artery ligation and PTH (80 µg/kg/d) was injected for 5 days afterwards. Number of BMCs was analyzed by flow cytometry. SDF-1 protein levels and activity of dipeptidylpeptidase IV (DPP-IV) were investigated by ELISA and activity assay. Functional analyses were performed at day 30 after MI. PTH treated animals revealed an enhanced homing of CXCR4+ BMCs associated with an increased protein level of the corresponding homing factor SDF-1 in ischemic myocardium. *In vitro* and *in vivo*, PTH inhibited the activity of DPP-IV, which cleaves and inactivates SDF-1. Functionally, PTH significantly improved myocardial function after MI. Both, stem cell homing as well as functional recovery were

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reversed by the CXCR4 antagonist AMD3100. Conclusion: In summary, PTH is a direct DPP-IV inhibitor leading to an increased cardiac SDF-1 level, which enhances recruitment of CXCR4+ BMCs into ischemic myocardium associated with attenuated ischemic cardiomyopathy. Since PTH is already clinically used our findings may have direct impact on the initiation of studies in patients with ischemic disorders.

Poster Board Number: 1098

INCREASED NUMBER OF CD133 POSITIVE CARDIAC PROGENITOR CELLS IN THE HEARTS OF PATIENTS WITH CONGENITAL HEART DISEASE: *IN VITRO* AND *IN VIVO* ASSESSMENT OF DIFFERENTIATION AND FUNCTIONALITY

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Since the late 90s, there has been several observations indicating that human cardiac cells divide after myocardial infarction; thereafter a considerable effort have been made to discover and characterize resident cardiac progenitor cells suitable for cardiac regeneration. Yet, no feasible method is developed for cardiac progenitor separation based on the expression of a specific surface antigen. In addition, none of the candidate antigens were proved to mark all progenitor populations residing in the heart. This work focuses on isolation and characterization of the progenitors present in tissue samples obtained from patients with atrial septal defect, ventricular septal defect and tetralogy of fallot. The assumption is that in these patients, general hypoxemia and structural defect in heart recruits and/or stimulates progenitors proliferation. In contrast to the previous reports on the inability of CD133+ hematopoietic stem cells in differentiating towards fully functional Cardiomyocytes, our results show that CD133 antigen is one of the surface markers of cardiac progenitor cells and the CD133 positive (CD133+) cells can form mature cardiomyocytes. Heart biopsies (100-300 mg) were obtained, with informed consent, from patients during their heart surgery. After enzymatic digestion of tissue samples, flow cytometric analysis revealed the presence of significant level of CD133+ cells in patients' samples (23.33 ± 8.88%). The Colony Forming Unit (CFU) assay confirmed that these cardiac resident CD133+ cells had the ability to generate hematopoietic lineages. Two different approaches were taken in order to examine the ability of the isolated CD133+ cells to differentiate towards cardiomyocytes. In the first method, the cells resulted from enzymatic tissue digestion were incubated for four days and then the expression level of CD133 antigen along with ISL-1 and MEF2C transcription factors were evaluated by flow cytometry during this period. During the first day, most of CD133+ cells were also ISL-1 positive with CD133/MEF2C double positive cells comprising only a small proportion of CD133+ cells. Interestingly, the reverse trend was observed on the fourth day. In the second method, the cells were incubated with CD133 conjugated microbeads and then CD133+ cells were sorted using a MACS device. The sorted cells were expanded subsequently. CD133+ cells were differentiated by adding 5-azacytidine for 3 days to culture medium. During the next 28 days, the expression profile of cardiac specific genes was determined by RT-PCR in different stages of differentiation. To confirm the data obtained by RT-PCR, immunofluorescence staining for myosin heavy chain, alpha cardiac actin, ISL-1, MEF2C and Nkx2.5 markers were also performed on differentiated cells. Electrophysiological recordings verified the functionality of differentiated cells. In order to get a better insight about the *in vivo* functionality of CD133+ cells, these cells were expanded and transplanted to rat infarct models. After six weeks, cardiac repair was evaluated using echocardiography and immunohistochemistry to evaluate the differentiation potential of CD133+ progenitor cells in rat heart. These studies revealed promising potential of CD133+ cells as a source for cardiac regeneration.

Poster Board Number: 1100

CARDIOMYOGENIC POTENTIAL OF CARDIOSPHERE-DERIVED CELLS

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The heart is no longer considered a post-mitotic organ since it has been demonstrated that cardiomyocyte renewal occurs during our entire lifespan. The cells responsible for heart regeneration are the cardiac stem cells. The objective of this work was to study the *in vitro* properties of human cardiosphere-derived cells (CDCs), one of the cardiac stem cell types described so far, and their ability to differentiate into cardiomyocytes. CDCs were obtained from human atrial myocardial tissue discarded after cardiac surgery by enzymatic digestion with Collagenase type II (0.4%). Ten to fifteen days after isolation, small round phase-bright cells (PBC) appeared on top of the fibroblast-like cells. PBC were collected and placed in a non-adherent plate for 2 days where they formed cardiospheres, which were then transferred to adherent plates, giving rise to CDCs. These cells are adherent to plastic and present a spindle-shaped morphology. Surface molecule expression was studied by flow cytometry. CDCs were positive for the mesenchymal stem cell (MSCs) markers CD105, CD90 and CD73 and negative for CD34, CD45, CD31 and all the hematopoietic lineage markers. Since CDCs expressed the core mesenchymal molecules found in bone marrow (BM) MSCs, we performed experiments to test their ability to differentiate in osteogenic and adipogenic lineages, a well-known property of BM MSCs. CDCs were unable to differentiate into these lineages. No numeric chromosome abnormalities were found in CDCs cultivated *in vitro* for 2 passages and these cells had a population doubling time of approximately 53.14 ± 5.63 hours. CDCs were then co-cultured for 72 hours with neonatal ventricular myocytes isolated from transgenic mice with the green fluorescent protein (GFP) gene under the control of the β-actin promoter. We detected the expression of connexin 43, troponin T, myosin light chain 2a and myosin heavy chain by RT-PCR, suggesting that these cells can be differentiated into cardiomyocytes. In conclusion, CDCs are cells of mesenchymal origin, adherent to plastic and immunophenotipically similar to BM-MSCs. However, their differentiation properties are quite different, as BM-MSCs differentiate in osteogenic, adipogenic and chondrogenic lineages, while CDCs differentiate into the cardiac lineage. It still remains to be defined if CDCs can differentiate into the other cell types found in the heart and if these cells have the ability to generate cardiomyocytes *in vivo*.

Poster Board Number: 1102

DUAL ACTION OF ANGIOPOIETIN-1 TO PROTECT HEART FROM ISCHEMIA/ REPERFUSION INJURY THROUGH VE-CADHERIN STABILIZATION AND INTEGRIN-B1-MEDIATED SURVIVAL SIGNAL

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Rationale: Early reperfusion after myocardial ischemia that is essential for tissue salvage also causes myocardial and vascular injury. Cardioprotec-

tion during reperfusion therapy is an essential component in treatment of myocardial infarction. Angiopoietin-1 has been known as an endothelial specific-angiogenic factor. The effects of angiopoietin-1 on cardiomyocytes and vascular cells under reperfusion condition have not been investigated. Objective: We propose a protective mechanism whereby angiopoietin-1 increases the integrity of the endothelial lining and it exerts a direct survival effect on cardiomyocytes under myocardial ischemia followed by reperfusion. Methods and Results: Firstly, we found that angiopoietin-1 prevented vascular leakage through regulating VE-cadherin phosphorylation. The membrane expression of VE-cadherin was markedly decreased on hypoxia/reoxygenation, which was recovered by angiopoietin-1. Interestingly, these effects were mediated by the facilitated binding between tyrosine phosphatase and VE-cadherin, leading to its de-phosphorylation. siRNA against tyrosine phosphatase abolished the effect of angiopoietin-1 on VE-cadherin de-phosphorylation, and thereby decreased levels of membrane-localized VE-cadherin. Secondly, we found angiopoietin-1 prevented cardiomyocytes death although they lack angiopoietin-1 receptor Tie2. Angiopoietin-1 increased cardiomyocytes survival through integrin-β1, leading to decrease of active caspase-9 and then reduction of active caspase-3. Neutralizing antibody against integrin-β1 blocked these protective effects of angiopoietin-1 on cardiomyocytes. In a mouse myocardial ischemia/reperfusion model, angiopoietin-1 enhanced cardiac function; reduction of left ventricular end-systolic dimension (LVESD) and left ventricular end diastolic dimension (LVEDD) with the increase of ejection fraction (EF) and fractional shortening (FS). Conclusion: Our findings suggest the novel cardioprotective mechanisms of angiopoietin-1 which are achieved by reductions in both vascular leakage and cardiomyocyte death after ischemia/reperfusion.

Poster Board Number: 1106

ADULT CARDIAC STEM CELLS POTENTIAL FOR CARDIAC TRANSPLANT PATIENTS

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Myocardial infarction is the leading cause of death among people in industrialized nations. Although the heart has some ability to regenerate after infarction, myocardial restoration is inadequate. In several studies, patients with coronary disease who received autologous bone marrow cells by intracoronary injection show significant 3.7% (range: 1.9-5.4%) increases in LV ejection fraction, decreases in LV end-systolic volume of -4.8 ml (range: -1.4 to -8.2 ml) and reductions in infarct size of 5.5% (-1.9 to -9.1%), without experiencing arrhythmias. Bone marrow cells appear to release biologically active factors that limit myocardial damage. Unfortunately, bone marrow cells from patients with chronic diseases propagate poorly and can die prematurely. In the present study we have identified a sub-population of adult human cardiac stem cells (HACSCs) that can be utilized for restoration of myocardium. Over the past decade, the ability to culture and differentiate human adult cardiac stem cells (HACSCs) has offered researchers a novel therapeutic that may, for the first time, repair regions of the damaged heart. Studies of cardiac development in lower organisms have led to identification of the transforming growth factor-β superfamily (eg, activin A and bone morphogenic protein 4) and the Wnt/β-catenin pathway as key inducers of mesoderm and cardiovascular differentiation. These factors act in a context-specific manner (eg, Wnt/β-catenin is required initially to form mesoderm but must be antagonized thereafter to make cardiac muscle). Different lines of HACSCs produce different levels of agonists and antagonists for these pathways, but with careful optimization, highly enriched populations of immature cardiomyocytes can be generated. These cardiomyocytes survive transplantation to infarcted hearts of experimental animals, where they create new human myocardial tissue and improve heart function. The grafts generated by HACSCs transplantation have been small, however, leading to an exploration of tissue engineering as an alternate strategy. Engineered tissue generated from preparations of adult human cardiomyocytes survives after transplantation may be because of recruitment of resident Cardiac stem cell signaling pathways. Creation of pre-organized vascular networks in the tissue markedly enhances survival, with human capillaries anastomosed to the host coronary circulation. Thus, pathways controlling formation of the human cardiovascular system are emerging, yielding the building blocks for tissue regeneration that may address the root causes of heart failure.

ENDOTHELIAL CELLS/HEMANGIOBLASTS

Poster Board Number: 1108

HEMANGIOBLASTIC POTENTIAL OF ADULT HUMAN CIRCULATING CD34-LIN-CD45-CD133-CELLS

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A precise identification of human adult hemangioblast with a clear-cut definition of characterizing molecular markers is still lacking. To possibly identify circulating precursors endowed with the hemangioblast developmental potentials, we established a new ex-vivo long-term culture model aimed to support the concomitant differentiation of hematopoietic and endothelial cell lineage. We identified a peripheral blood population lacking the expression of CD34, lineage markers, CD45 and CD133 (CD34-Lin-CD45-CD133- cells) endowed with the ability to differentiate after 6-week culture into both hematopoietic and endothelial lineages. We demonstrated the bilineage potentiality of CD34-Lin-CD45-CD133-cells at the single cell level, and showed that CD34- Lin-CD45-CD133- cells transplanted in NOD/SCID mice reconstitute the hematopoietic tissue and generate functional endothelial cells that contribute to new vessel formation during tumour angiogenesis, thus confirming the bilineage potentiality of these cells *in vivo*. Further molecular and functional characterization will aid the comprehension of the possible physiologic role of CD34-Lin-CD45-CD133- cells in blood and blood vessel maintenance and repair in adult life.

Poster Board Number: 1110

PLURIPOTENT MODELS OF PULMONARY ARTERIAL HYPERTENSION: INVESTIGATING THE EFFECTS OF BMP DYSREGULATION ON VASCULAR DIFFERENTIATION USING HUMAN IPS CELLS

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Idiopathic pulmonary arterial hypertension (PAH) is a debilitating condition asscociated with extensive remodelling of the pulmonary vasculature, which causes increased pulmonary vascular resistance and eventually right heart failure. The life-expectancy of a patient diagnosed with idiopathic PAH is less than three years, during which time increasing lung dysfunction severely impinges upon quality of life. At the cellular level, morphological aberrations are apparent in both the endothelial and smooth muscle layers of the small

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peripheral arteries in the lungs of PAH patients. Dysregulated cell proliferation leads to increased muscularisation of these vessels and ultimately occlusion of the lumen. At the molecular level, autosomal dominant mutations in the type II BMP receptor, BMPR2, have been detected in the majority of cases of familial PAH. Mutations in this gene have also been detected in patients with non-inherited, sporadic PAH, implicating dysregulated BMP signalling as a crucial factor in the development of this pulmonary pathology. Mechanistic analysis of the molecular basis of PAH is hindered by the limited supply of patient-derived endothelial and smooth muscle cells, and further exacerbated by the poor proliferative capacity of these cell populations. To overcome this constraint, we are generating iPS cell lines from patients harbouring a variety of mutations in BMPR2. We have already established iPS cell lines from both a PAH patient with the null mutation W9X and from their asymptomatic cousin, who also possesses this mutant allele. We are currently characterising the differentiation potential of these cell lines in both embryoid bodies and in chemically defined adherent culture conditions. As BMP signalling is important in ventral mesoderm specification, we will assess the potential of PAH mutant iPS cells to adopt this fate in response to ligand stimulation. We are particularly interested in determining the ability of PAH iPS cells to acquire endothelial and smooth muscle identities, since PAH is attributed to abnormalities in these cell types. To investigate this, we isolate CD34+ve PAH cells and direct their differentiation into endothelial and smooth muscle lineages, assessing functionality by a range of assays, including expression of differentiation markers and the ability of endothelial cells to form networks *in vitro*.

Poster Board Number: 1112

LARGE-SCALE DERIVATION OF ENDOTHELIAL PROGENITOR CELLS FROM HUMAN EMBRYONIC STEM CELLS USING CHEMICALLY DEFINED CULTURE CONDITIONS

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Most solid tumors eventually require the formation of neo-vasculature for continued growth and metastases. This dependence on angiogenesis has been exploited in anti-cancer therapies with monoclonal antibodies, small molecule inhibitors, and cell-based approaches. Among the latter strategies is a so-called Trojan horse approach that includes *ex vivo* derivation and "arming" of tumor-homing cells followed by their systemic delivery and release of a toxic payload at the tumor. As a first step toward such a Trojan horse approach, we have established a highly efficient process for the derivation of endothelial progenitor cells (EPCs) from human embryonic stem cells (hESCs). Our process to date has reproducibly provided high cell yields (>4x10^8 cells) and purities (approaching 99%) within 2-3 weeks of culture initiation. These cells display phenotypic (morphology, cell surface antigen, and gene expression) and functional vascular endothelial cell characteristics (tube formation *in vitro* and incorporation into both normal and tumor neo-vasculature *in vivo*). Our derivation process has been applied to 5 independent hESC lines, including H1 (WA01) and H9 (WA09), as well as 3 of our GMP-compliant hESC lines (ESI 017, ESI 035 and ESI 051). The process uses a chemically-defined medium with sequential additions of recombinant human cytokines and signaling inhibitors, incorporates an optional immunomagnetic sorting step for CD31 or other characteristic markers, and is scalable to clinical demands. It does not involve feeder cells, serum, or xenogeneic components. Furthermore, our EPCs can be cryopreserved and banked, and are recovered with high efficiencies and the preservation of endothelial phenotypic characteristics.

Poster Board Number: 1114

DERIVATION OF CLONAL ENDOTHELIAL PROGENITOR CELLS FROM HUMAN PLURIPOTENT STEM CELLS

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Endothelial dysfunction with resulting atherosclerosis is a fundamental cause for vascular injury in cardiovascular patients. Increasing evidence suggests that circulating endothelial progenitor cells (EPCs) monitor the health of the endothelium and are recruited into a site of vascular injury or tissue ischemia to orchestrate the initiation of the angiogenic response. Endothelial colony forming cells (ECFCs) are subtype of EPCs with robust clonal proliferative potential that display intrinsic *in vivo* vessel forming ability. However, ECFCs are rare in number (1/107-108 peripheral blood mononuclear cells) and their number tends to decline with age or develop replicative senescence. In elderly patients and subjects with cardiovascular disease, these cells may become prone to replicative senescence lacking proliferative potential, thus, rendering them impotent for vascular repair. Thus, generation of patient-specific induced pluripotent stem (iPS) cells from cardiovascular patients and then deriving autologous ECFCs from the iPS cells for repair of their own damaged blood vessels may be a viable treatment solution. Directed differentiation of fibroblast-derived human iPS cells generates autologous endothelial cells that have not yet been fully characterized. It is unclear whether human iPS cell-derived endothelial cells possess a robust proliferative potential and *in vivo* vessel forming ability as displayed by umbilical cord blood- or adult peripheral blood-derived ECFCs. Further, factors and mechanisms that control the differentiation of human PS (human iPS and human ES) cells into endothelial cells with ECFC properties are completely unknown. Therefore, we sought to develop an endothelial differentiation protocol that would allow us to derive and isolate endothelial cells with an ECFC phenotype and function. Endothelial lineage differentiation was initiated by growing human pluripotent cell-derived embryoid bodies (EBs) in suspension culture for 4 days and seeding them on gelatin or matrigel coated dishes for another 10 days. On day 14, cells were harvested and sorted using antibodies that recognize endothelial antigens CD31, CD144, CD146, and NRP-1. Sorted cells were subsequently cultured in endothelial growth (EGM-2) media supplemented with TGFβ inhibitor (10 μM SB431542) for 2 weeks. These cells formed a homogenous monolayer with characteristic cobblestone appearance, exhibited clonal proliferative potential at a single cell level, and demonstrated angiogenic behavior by forming capillary like structures when cultured in matrigel. These cells also formed capillary like structures in cellular collagen gels implanted into the flanks of NOD/SCID mice and we are currently verifying these implants for their presence of humanized blood vessels. Thus, human pluripotent stem cells may be differentiated into cells with ECFC properties, leading the way for pre-clinical studies of their function.

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HUMAN UMBILICAL CORD BLOOD ALDH-HIGH PROGENITOR CELLS PROMOTE A PRO-ANGIOGENIC PARACRINE MICROENVIRONMENT

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Angiogenesis is a critical process for tissue repair and regeneration. Understanding the role of somatic progenitor cells in angiogenesis is integral to the development of effective revascularization therapies. Human umbilical cord blood (UCB) represents a readily available allogeneic source of functional pro-angiogenic progenitor cells for the development of cell-based strategies to treat ischemic disease. Human UCB cells can be purified by high aldehyde dehydrogenase (ALDH) activity, a conserved function in multiple progenitor cell lineages. Fluorescence-activated cell sorting accrues clinically applicable cell populations with high (ALDHhi=0.4±0.1%) versus low (ALDHlo=41.2±3.6%) ALDH activity (n=10). Whereas UCB ALDHlo cells are devoid of progenitor cell function *in vitro*, ALDHhi cells were highly enriched for hematopoietic (1 in 4 cells, n=4) and endothelial (1 in 5x104 cells, n=4) colony forming cells. While mature endothelial cells (ECs) plated in growth factor-free media rapidly die, supplementation with ALDHhi cells in transwell inserts promotes EC survival and expansion without cell contact (n=3, $p<0.05$). Furthermore, in direct co-culture, human UCB ALDHhi cells augment the tubule forming capacity of mature ECs plated on matrigel (n=6, $p<0.05$). *In vivo*, the vascular regenerative capacity of UCB ALDH-purified cells was assessed by tail-vein transplantation into NOD/SCID mice with acute hindlimb ischemia induced by unilateral femoral artery ligation and excision. Laser Doppler perfusion imaging was used to track the recovery of blood flow indicated by the perfusion ratio (PR) of the ischemic versus non-ischemic limb. Mice transplanted with UCB ALDHhi cells showed significantly improved recovery of limb perfusion by day 14 post-transplantation (PR=0.58±0.07, n=7) compared to mice injected with saline vehicle control (PR=0.28±0.03, n=6, $p<0.01$). Mice transplanted with ALDHhi cells showed specific recruitment to the ischemic hindlimb 3 and 7 days after transplantation as detected by flow cytometry and histochemistry for human cells in muscle sections. Notably, this robust recovery of blood flow to the ischemic limb was maintained stably despite diminished engraftment of human cells by 28 days post-transplantation (n=6, $p<0.01$). Sublethal irradiation (275cGy) of recipient mice to enhance engraftment of human UCB ALDHhi cells was less effective and only supported a transient recovery of blood flow compared to saline controls, as initial cell recruitment was diverted to the bone marrow. Although significantly improved perfusion was not maintained in irradiated mice, ischemic limbs of ALDHhi-transplanted mice also showed increased capillary densities compared to all other treatments at day 28 ($P<0.01$) quantified by enumeration of von Willebrand factor+ blood vessels, confirming the pro-angiogenic potential of UCB ALDHhi cells *in vivo*. Collectively, these findings show that human UCB-derived ALDHhi cells can actively recruit to areas of ischemic injury where they promote a pro-angiogenic microenvironment by paracrine support of ECs, accelerating the recovery of blood flow and enhancing endogenous revascularization. As a readily available population of pro-angiogenic cells, UCB ALDHhi mixed progenitor cells may prove useful in the development of allogeneic cell-based clinical therapies for ischemic disease.

Poster Board Number: 1118

HEPARAN SULFATE PROTEOGLYCAN MEDIATES SHEAR STRESS-INDUCED ENDOTHELIAL GENE EXPRESSION IN MOUSE EMBRYONIC STEM CELL-DERIVED ENDOTHELIAL CELLS (ESC-DERIVED CD31+/CD45- CELLS)

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It has been shown that the shear stress plays a critical role in promoting endothelial cell (EC) differentiation from embryonic stem cell (ESC)-derived EC. However, the underlying mechanisms mediating shear stress effect in this process have yet to be investigated. It has been reported that the glycocalyx component heparan sulfate proteoglycan (HSPG) mediates shear stress mechanotransduction in mature EC. In this study, we investigated whether cell surface HSPG plays a role in shear stress modulation of EC phenotype. ESC-derived endothelial cells were subjected to shear stress (5 dyn/cm2) for 8 hours with or without heparinase III (Hep III) that digest heparan sulfate. Immunostaining showed that ESC-derived EC surfaces contain abundant HSPG, which could be cleaved by Hep III. We observed that shear stress significantly increased the expression of the vascular endothelial cell-specific markers genes (vWF, VE-cadherin, PECAM-1). The effect of shear stress on expression of tight junction genes (ZO-1, OCLD, CLD5) was also evaluated. Shear stress increased the expression of ZO-1 and CLD5, while it didn't alter the expression of OCLD. Shear stress increased expression of vasodilatory gene (eNOS, COX-2) expression, while it decreased the expression of the vasoconstrictive gene ET1. After reduction of HSPG with Hep III, the shear stress-induced expression of vWF, VE-cadherin, ZO-1, eNOS, and COX-2, were abolished, suggesting that shear stress-induced expression of these genes depends on HSPG. These findings indicate for the first time that HSPG is a mechanosensor mediating shear stress-induced EC maturation from ESC-derived EC cells.

Poster Board Number: 1120

PROMINENT ROLE OF RESIDENT ENDOTHELIAL PROGENITOR CELLS DURING WOUND HEALING IN MICE

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Endothelial progenitor cells (EPCs) are central to the formation of new vessels that occurs during tissue repair and are attractive candidates for regenerative cell therapies. However, current knowledge regarding their immuno-phenotype and reservoirs is inadequate and controversial. We sought to quantitate the respective roles of skin-resident and bone marrow-derived putative EPCs during wound healing to determine hierarchy and origin of the cells participating. Excisional wounds were generated on C57BL/6 mice and collected daily from day 1 to 5 after wounding. All tissues were analysed using 5-colour flow cytometry for CD45, CD11b, CD34, CD31 and VEGFR2. Unbiased analyses were performed to establish hierarchy. Absolute number modifications in each compartment (wound, blood, bone marrow) were calculated and considered for potential exchange between compartments. Here, we propose a cell population defined as CD34+CD45loCD31loVEGFR2lo that exists as an omnipresent EPC. In skin wounds these putative EPCs proliferated from day 1 and exhibited peak expansion on day 4 of healing. Within this expanded pool, a population of CD34+CD45loCD31hiVEGFR2hi cells appeared from day 2 and increased 3 fold in absolute number between day 4 and 5 post wounding, suggest-

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ing terminal differentiation of progenitors. The contribution of EPCs or terminally differentiated cells from bone marrow or the circulating blood was quantitatively small and occurred later than this initial local response. We propose that putative EPCs exist in local rather than distant pools and proliferate to give rise to the neovascularisation. These data challenge the current paradigm for EPC recruitment during wound healing and provide invaluable insight into a useful but poorly understood tissue progenitor population.

Poster Board Number: 1122

BMP9 INDUCES PROLIFERATION OF MOUSE EMBRYONIC STEM CELL-DERIVED ENDOTHELIAL CELLS

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Members of the bone morphogenetic protein (BMP) family have been implicated in the development and maintenance of vascular systems. While members of the BMP2/4 and osteogenic protein-1 groups signal via activin receptor-like kinases (ALK)-2, 3, and 6, BMP9 and 10 have been reported to bind to ALK-1 in endothelial cells. However, the roles of BMP9/ALK-1 signaling in the regulation of endothelial cells have not yet been fully elucidated. Here, we examined the effects of BMP9 on the proliferation of endothelial cells using various systems. Vascular tube formation from *ex vivo* allantoic explants of mouse embryos was promoted by BMP9. BMP9, as well as BMP4 and 6, also induced the proliferation of *in vitro*-cultured mouse embryonic stem cell-derived endothelial cells (MESEC) by inducing the expression of vascular endothelial growth factor receptor 2 and Tie2, a receptor for Angiopoietin-1. Decrease in ALK-1 expression and expression of constitutively active ALK-1 in MESEC abrogated and mimicked the effects of BMP9 on the proliferation of MESEC, respectively, suggesting that BMP9 promotes their proliferation via ALK-1. Furthermore, *in vivo* angiogenesis was promoted by BMP9 in a Matrigel plug assay and a BxPC3 human pancreatic cancer xenograft model. Furthermore, we found that BMP9 inhibited *in vivo* lymphangiogenesis in a BxPC3 human pancreatic cancer xenograft model. These findings suggest that BMP9 signaling activates the endothelium tested in the present study via ALK-1.

Poster Board Number: 1124

IN VITRO VESSEL FORMING CAPACITY OF ENDOTHELIAL PROGENITOR CELLS IN HYPERGLYCEMIC CONDITIONS

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In type 2 diabetes the impairment of vascular repair processes and angiogenesis due to endothelial progenitor cell (EPC) dysfunction can be improved after strict glycemic control. We established an *in vitro* vessel formation assay of EPCs by using CFSE staining system in order to study the effects of hyperglycemic conditions. This system quantifies the vessel forming capacity of CFSE-labeled EPCs cultured in hyperglycemic conditions, with good glycemic control (189.5 mg/dl of D-glucose) or poor glycemic control (295.5 mg/dl of D-glucose) compared to normal glycemic condition (100 mg/dl of D-glucose) when co-culturing with human umbilical vein endothelial cells (HUVECs). From three independent experiments, we found that the *in vitro* vessel forming capacity was impaired in EPCs cultured in hyperglycemic conditions compared to normal glycemic conditions (43.40 ± 0.77% for good glycemic condition, 34.70 ± 0.70% for poor glycemic condition and 50.78 ± 2.07% for normal glycemic conditions; $p < 0.01$). We studied the gene expression profiles of 15 candidate genes involving in vessel formation and found that there was a lower gene expression level only for angiopoietin1 whereas there was a higher gene expression level for VECAM1 in EPCs cultured in hyperglycemic conditions compared to normal glycemic condition. The results were validated by coculturing EPCs in hyperglycemic conditions with recombinant angiopoietin1 which showed a significant increase in vessel forming capacity of EPCs in hyperglycemic condition (35.33 ± 1.98% to 48.76 ± 2.67%, $p< 0.05$). In contrast, angiopoietin2 (a competitive inhibitor of angiopoietin1) was shown to inhibit the vessel forming capacity of EPCs cultured in normal glycemic condition (51.83 ± 1.32% to 41.28 ± 0.63% , $p< 0.01$). We conclude that *in vitro* vessel forming capacity of EPCs in hyperglycemic condition is impaired and angiopoietin1 may play an important role in the vessel forming capacity of EPCs.

Poster Board Number: 1126

DIRECT AND DIFFERENTIAL EFFECTS OF STEM CELL FACTOR ON THE NEOVASCULARIZATION ACTIVITY OF ENDOTHELIAL PROGENITOR CELLS

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Aims Most previous studies on the role of SCF in EPC-mediated neovascularization have focused on the EPC mobilization and homing process. However, the direct effects of SCF on neovascularization activity of EPCs have not been characterized. We sought to determine whether SCF regulates the neovascularization ability of EPCs by comparing its roles in mature endothelial cells. Methods and Results *In vitro* and *in vivo* assays revealed that SCF substantially increased the neovascularization activity of human EPCs through the SCF receptor c-Kit. Notably, the SCF-induced increase in neovascularization activity was substantially greater in EPCs than that in HUVECs. SCF-induced phosphorylation of c-Kit and its downstream signaling molecules was consistently found to be more potent and longer-lasting in EPCs than in HUVECs. This high responsiveness of EPCs to SCF was explained by the finding that the cell-surface expression of c-Kit is far higher in EPCs than in HUVECs. A c-Kit promoter assay revealed that the increased expression of c-Kit in EPCs could be attributed to the greater expression of Scl, LMO2, and GATA2. Conclusion In addition to its documented role in the mobilization and recruitment of EPCs, our findings show that SCF directly enhances the neovascularization activity of EPCs. Furthermore, the present study provides further evidence that EPCs exhibit differentially greater responsiveness to hypoxia-inducible cytokines including SCF than mature endothelial cells, suggesting that EPCs in ischemic tissues function differently from mature endothelial cells, although they exhibit very similar phenotypes.

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Poster Board Number: 1128

TRANSPLANTATION OF AUTOLOGOUS ENDOTHELIAL PROGENITOR CELLS IMPROVES PULMONARY ENDOTHELIAL FUNCTION AND GAS EXCHANGE IN RABBITS WITH ENDOTOXIN-INDUCED ACUTE LUNG INJURY

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Background. Circulating endothelial progenitor cells (EPCs) have been therapeutically applied to aid vascular repair and myocardial regeneration. The number of circulating EPCs also provides invaluable outcome prediction for fatal diseases such as acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). However, evidence for the therapeutic potential of EPCs in subjects with ALI/ADRS is limited. Methods. Circulating EPCs were obtained from rabbits using Ficoll centrifugation. One week after culturing EPCs in endothelial growth medium-2, ALI was induced in rabbits by intratracheal instillation of lipopolysaccharide (LPS, 500 μg/kg). Autologous EPCs or saline were administered intravenously following induction of ALI and animals were sacrificed 2 days later. Pulmonary artery endothelial function and gas exchange were determined. Degrees of lung injury were assessed by alveolocapillary permeability, lung hemoglobin content and myeloperoxidase (MPO) activity. Results. Compared with controls, partial pressure of oxygen in arterial blood was significantly elevated and pulmonary artery endothelium-dependent relaxation response was restored in rabbits receiving EPC transplantation. Lung water, Evan's blue and bronchoalveolar lavage protein contents were significantly reduced in EPC transplanted group, indicating a better preservation of the alveolocapillary membrane. Transplantation of EPCs decreased lung hemoglobin level. Furthermore, expressions of CD11b and MPO activity were also suppressed following administration of EPCs. Conclusions. Transplantation of EPCs restored pulmonary endothelial function, preserved integrity of alveolocapillary barrier and suppressed lung inflammatory response, thereby improving pulmonary gas exchange in rabbits with intratracheal LPS-induced ALI. Transplantation of EPCs can be a novel cell-based, endothelium-targeted therapeutic strategy for prevention and treatment of ALI/ARDS.

Poster Board Number: 1130

WHAT'S THE ORIGIN OF AN OUTGROWTH ENDOTHELIAL PROGENITOR CELL?

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Background: The observation that peripheral blood contains a population of cells capable of forming endothelial cells *in vitro* and contributing to angiogenesis *in vivo* suggested the presence of a circulating endothelial progenitor cell (EPC). A decade of research has sought to identify the origin and phenotype of EPC and to harness their potential for cardiovascular regeneration. Whilst this concept is clearly important, ambiguity regarding the precise definition of an EPC has hampered progress in this field. Of the populations identified so far, endothelial outgrowth cells (EOC) most closely fulfil the criteria of an EPC, but their origin and circulating precursor have not been identified. Methods and Results: Using enrichment and depletion strategies to isolate cell fractions based on the expression of stem cell and endothelial markers, we provide evidence that EOC potential is associated with the CD34+CD133-CD146+ cell fraction. Mature endothelial cells are also defined as CD34+CD133-CD146+ cells. We have further investigated the origin of EOC and compared them to mature human umbilical vein endothelial cells (HUVEC). We report that EOC are identical to mature HUVECs by morphology, surface antigen expression, immunohistochemistry, RT-PCR, proliferation and functional assessments. *In vivo* data show that early passage EOC and HUVEC implanted in a mouse model of angiogenesis gave substantial blood vessel formation incorporating cells of human origin. On the other hand, our results showed that whilst EOC can be readily isolated from umbilical cord and peripheral blood they cannot be isolated from bone marrow or G-CSF mobilised blood raising the possibility that the precursors of EOC are derived from an alternative stem cell niche outside the bone marrow. Indeed, BM-derived outgrowth cells were positive for mesenchymal markers, negative for endothelial markers and they did not form blood vessels *in vivo*. Conclusion: Our study demonstrated the similar, if not identical, nature of EOC to mature endothelial cells and questions the source of these cells. The site of origin of EOC is presently unknown but the current literature suggests that human circulating endothelial progenitors appear to be bone marrow (BM) derived. In our study, BM had negligible numbers of CD34+CD133-CD146+ cells and lacked the potential to generate EOC colonies. We therefore conclude that bone marrow is not a source of EOC.

Poster Board Number: 1132

ROLE OF EPC IN VASCULARISED BONE TISSUE ENGINEERING

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Clinical translation of bone tissue engineering approaches for fracture repair has been hampered by the lack of adequate vascularization required for maintaining cell survival and skeletal regeneration. Here, we investigated the vasculogenic and osteogenic contributions of umbilical cord blood derived endothelial progenitor cells (EPC) when cocultured with human fetal mesenchymal stem cell (hfMSC) in both *in vitro* and *in vivo* paradigms for the generation of vascularised bone grafts. It is hypothesised that the addition of EPC to osteogenic-primed hfMSC constructs will lead to enhanced vascularisation and osteogenesis, eventually accelerating fracture healing. Coculture of EPC enhanced hfMSC osteogenic differentiation, resulting in earlier induction of ALP (Day 7 versus Day 10) and increased calcium deposition (1.9X; $p<0.001$) compared to hfMSC alone. This effect was observed only in bone inductive medium, but not in basal medium, suggesting that EPC does not induce osteogenic programming by itself. Instead, the soluble factors secreted by EPC was found to be responsible for this observed osteogenic enhancement, which led to 1.8-2.2X higher ALP levels and 1.4-1.5X increase in extracellular calcium deposition ($p<0.01$) when hfMSC were cultured in EPC conditioned media (EPCCM) compared to bone media culture. Protein analysis of the EPCCM revealed a wide spectrum of secreted proteins containing pro-inflammatory cytokines, angiogenic factors and bone-related proteins such as Angiogenin, Angiopoietin-2, BMPs and TGF-βs. To observe the cellular distribution in coculture, cells were dual fluorescent-labelled by lentiviral vectors. Islet formation by EPC and subsequent development into vessel-like structures over time was observed only in coculture with hfMSC but not in EPC monoculture alone. Translation of the coculture from two-dimensional monolayer into three-dimensional scaffold constructs also supported an extensive EPC-derived vessel-like network within the microarchitecture of the scaffold *in vitro*. To investigate the performance of EPC/hfMSC coculture *in vivo*, cellular-scaffold constructs were implanted subcutaneously in immunodeficient mice. Microfil perfusion revealed extensive vascularisation penetration into the scaffolds, with the presence of human specific CD31+ lumenal-like structures on Week 3 in EPC/hfMSC-constructs only. This was accompanied by an increase in ectopic bone formation on Week 8 in the EPC/hfMSC constructs as compared to the hfMSC alone, suggesting the importance of vascularisation in aiding bone formation. This study highlighted the potency of umbilical cord blood-EPC as a potential vasculogenic and osteogenic-enhancing cell source

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for stem cell therapy in bone tissue engineering applications. Evidence of its mechanistic influence via its secreted paracrine modulators also suggests the possibility of use of its soluble factors as a therapeutic off-the-shelf biologics strategy for bone repair.

Poster Board Number: 1134

EFFECT OF IONIZING RADIATION INDUCED DAMAGE OF ENDOTHELIAL PROGENITOR CELLS IN VASCULAR REGENERATION

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Accumulated studies have revealed that roles of stress signalings, and subsequent stress response in stem/progenitor cells, is responsible for retarded regeneration or degenerative disease. As ionizing radiation (IR), which sensitizes diverse types of stem/progenitor cells, has been reported to induce cardio-circulatory diseases, we hypothesized that IR induced vascular abnormality is associated to defect of stem/progenitor cells that are responsible for vascular homeostasis, mainly endothelial progenitor cells (EPCs). Herein, we used the irradiated mouse model to mimic the IR effect on vasculorigenesis. Mouse EPCs isolated from irradiated mice and human EPCs exposed to IR were used for functional analysis and gene expression study. We showed the evidence that EPCs were depleted and their function for vasculorigenesis *in vitro* and *in vivo* was significantly reduced by a single IR exposure. Such IR mediated stress responses in EPCs, upregulating p21Cip1 and downregulating vascular endothelial growth factor (VEGF) is mediated by p53 transcriptional activity. These results suggest that suppression of p53 would be clinically applicable to minimize the functional defect of EPCs in order to prevent onset of vascular diseases from radio-therapy or radiation exposure. Conclusion: These studies provide a novel insight into addressing the mechanism of IR induced vascular damage.

Poster Board Number: 1136

IDENTIFICATION OF A NOVEL MICRORNA REGULATING ANGIOGENIC RESPONSES

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MicroRNAs are a class of small RNAs that regulate target gene expression on the post-transcriptional level. Several microRNAs, such as mir-126 or miR-221/222 have been reported to control angiogenesis. We previously developed a novel embryonic stem (ES) cell differentiation system that exhibits vascular cell differentiation and early vascular development using vascular endothelial growth factor (VEGF) receptor-2 (VEGFR2/Flk1)-positive cells as common progenitors. With the use of this system, we have been elucidating various cellular and molecular mechanisms of vascular cell differentiation, such as enhancement of EC differentiation from Flk1+ cells with protein kinase A activation and arterial EC specification through direct interaction of Notch and β-catenin signaling downstream of cAMP. In this study, to elucidate roles of microRNAs in EC differentiation and vascular formation, we screened microRNAs specifically expressed during EC differentiation with the use of our ES cell differentiation system. Then, we identified a microRNA (mir-X) that is expressed in vascular ECs from ES cells, more predominantly in arterial ECs than venous ECs. Mir-X is expressed also in the dorsal aorta of mouse embryo and in human EC lines. Over-expression of mir-X in human EC lines showed an inhibitory effect on angiogenic responses such as EC migration or tube formation. Mir-X is, thus, supposed to be an endogenous regulator of angiogenesis, suggesting its great possibility for clinical application in the treatment of cancer or ischemic diseases.

Poster Board Number: 1138

GENETIC ENGINEERING OF ENDOTHELIAL PROGENITOR CELLS WITH ENDOTHELIAL NITRIC OXIDE SYNTHASE IMPROVES ANGIOGENESIS IN RABBITS WITH HINDLIMB ISCHEMIA

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Background: The present study explored the effect of endothelial nitric oxide synthase (eNOS) gene transfer on the angiogenic potential of *ex vivo* expanded endothelial progenitor cells (EPCs) in a rabbit model of hindlimb ischemia. Methods: Rabbit peripheral blood EPCs were cultured and transfected with mammalian expression vector pcDNA3.1-eNOS containing full-length human eNOS gene. Ischemia was induced in the right hind limb of three groups of rabbits by ligation of the distal external iliac artery and excision of the common and superficial femoral arteries. In one group of animals, ten days after the surgery, autologous eNOS-EPCs were transplanted intramuscularly in the ischemic limb. Two other groups received an equivalent number of unmodified EPCs or phosphate buffered saline (PBS) respectively. Results: Two weeks after cell transplantation, the *in vivo* expression of eNOS was detected in limb tissue sections of eNOS-EPCs treated animals. Animals treated with eNOS-EPCs had a significant reduction in ischemic muscle necrosis and inflammation, augmentation in the capillary density ($P< 0.05$) and angiographic scores demonstrating distal arterial reconstitution and enhanced angiogenesis in comparison to animals transplanted with EPCs or PBS ($P< 0.05$). Conclusion: We conclude that modification of EPCs by eNOS constitutes an effective strategy to improve the efficacy of EPCs for therapeutic angiogenesis.

Poster Board Number: 1140

THE ROLE OF MESENCHYMAL STEM CELL CONDITIONED MEDIUM ON THE MIGRATION AND PROLIFERATION OF ENDOTHELIAL PROGENITOR CELLS

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Mesenchymal stem cells (MSCs) are multipotent stem cells, which have been regarded as potential cell sources for tissue replacement therapy. Although the ability of MSCs in restoring the damaged tissue by direct differentiation to generate specific cell types is still controversial, their role as a source of pro-angiogenic and anti-inflammatory cytokines is well established. There are several reports showing the positive effects of MSC-derived cytokines in ameliorating the symptom of ischemic heart disease and cerebrovascular

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disease in animal models. Recently, endothelial progenitor cells (EPCs) have been identified as an important regulator of vascular homeostasis and the major contributor of postnatal neovascularization. According to this, there might be an interaction between MSC-derived angiogenic cytokines and EPC function, which results in the neovascularization of ischemic tissues. In this study, the effect of cytokines secreted from bone marrow-derived MSCs (BM-MSCs) on the proliferation, migration and invasion of EPCs were investigated using an *in vitro* model. To investigate the effect of BM-MSCs on EPC proliferation, the conditioned medium from passage 4 of MSCs was collected and used for EPC culture. The results showed that the conditioned medium from BM-MSCs can enhance the proliferation of cultured EPCs compared with controls. In addition, the BM-MSCs were co-cultured with EPCs through the transwell system to assess their paracrine effects on EPC migration and invasion. The result showed that BM-MSCs can induce higher levels of EPC migration and invasion compared with controls. In conclusion, this study demonstrated that the factors secreted by BM-MSCs play an important role in the proliferation, migration and invasion of EPCs.

EPIDERMAL CELLS

Poster Board Number: 1142

FEEDER LAYERS COMPARISON FOR THE PRESERVATION OF THE PROLIFERATIVE CAPACITY OF HUMAN EPITHELIAL STEM CELLS GROWN *IN VITRO*

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INTRODUCTION: Epidermal cell sheets grown *in vitro* are used as skin substitutes in the treatment of severe burn wounds. Using a lethally irradiated fibroblast feeder layer allows for a better lifetime survival of human epithelial stem cells grown *in vitro*. Our team has already shown that mouse feeder layer mediated survival of keratinocytes (skin epithelial cells) comes in part from the stabilisation of the positive transcription factor Sp1 in keratinocytes. The goal of this project was to analyse the influence of co-culturing a human fibroblast feeder layers on the proliferation and differentiation of skin keratinocytes and determine whether it enhances the quality of epidermal cell cultures.
METHODS: Keratinocytes were cultured either alone or in the presence of a lethally irradiated feeder layer (mouse or human) for about twenty passages or until differentiation. Proteins, RNA and cell extracts were obtained at each passages. Sp1 expression levels were analysed at each passage by Western Blot. RESULTS: Both human and mouse feeder layers had similar influences on keratinocytes growth and differentiation as revealed by the number of passages reached by these cells before they became differentiated and by their corresponding levels of Sp1 expression. Keratinocytes were cultured up to passage 18, both with human and mouse fibroblast feeder layer, without major degradation of Sp1, as opposed to keratinocytes cultured alone that only reached passage 8 before entering terminal differentiation. Furthermore, Sp1 level started to decline at higher passages with human feeder layers as compared to mouse feeder layers in 4 out of 6 cell lines. CONCLUSION: Human feeder layers promote proliferation of keratinocytes and stabilize expression of Sp1. Studying the mechanisms by which feeder layers promote cell proliferation will allow us to enhance the quality of tissue grafts, such as those from both the skin and the cornea that are used in the treatment of burn wounds.

Poster Board Number: 1144

FIBROBLAST DIFFERENTIATION FROM HUMAN INDUCED PLURIPOTENT STEM CELLS IS LINKED TO EPIGENETIC REGULATION OF PDGFR-B AND ACQUISITION OF MESENCHYMAL FUNCTION

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Advances in human induced pluripotent stem (hiPS) cell biology have raised new questions regarding the effect of reprogramming on the phenotype and function of a variety of cell types differentiated from them. We recently derived cells from hiPS showing characteristic properties of stromal fibroblasts (iPDK) that were similar to human embryonic stem cell- (hES) derived fibroblasts (EDK), and contributed to normal skin development and repair in an *in vitro* 3D tissue. The goal of the current study was to characterize the epigenetic profile and associated functional properties hiPS-derived cells during their differentiation to compare them to input fibroblasts initially used for reprogramming. We performed a detailed methylation analysis to compare iPDK cells to input fibroblasts, and showed that iPDK cells shared many of the same CpG methylation sites as the original fibroblasts, and were distinct from hiPS and hES cells. Specifically, our analysis revealed that the promoter for platelet-derived growth factor-β (PDGFRβ), an important receptor in fibroblast lineage commitment and function, was demethylated following differentiation, and PDGFRβ gene expression was concomitantly increased in differentiated fibroblasts. Following knockdown of PDGFRβ receptor using shRNA, iPDK and EDK exhibited a phenotypic shift towards a mesenchymal phenotype, including downregulation of cytoskeletal genes such as alpha-smooth muscle actin and cytokeratin 18, as well as a decrease in expression of extracellular matrix proteins such as Type I Collagen and Tenascin C. We are currently studying the effects of PDGFRβ knockdown in a 3D tissue context to determine the importance of this receptor on the regenerative potential of iPDK cells. Our findings implicate the loss of methylation of the PDGFRB promoter during differentiation of hIPS cells as an important regulatory step in the commitment of fibroblast-like iPDK cells that may influence their phenotype and function.

Poster Board Number: 1146

DSG3 SAFEGUARDS MOUSE BULGE STEM CELL QUIESCENCE AND ITS LOSS RESULTS IN NICHE REPAIR AT THE EXPENSE OF HAIR FOLLICLE REGENERATION

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The stem cell (SC) niche is considered to govern SC homeostasis and repair. Cell-cell and cell-matrix adhesion is important in epithelial tissue remodeling through both structural and signaling-dependent mechanisms. Recent discussions about the contribution of cadherin-mediated niche adhesion in hematopoietic SC homeostasis and the proposition to eliminate quiescent cancer SC by targeting cadherins, emphasize an important role of cadherins in the SC niche. In skin, limited knowledge is available on mechanisms of SC activation during repair and specifically following loss of cadherin-mediated niche adhesion. Pemphigus vulgaris (PV) is a severe autoimmune blistering disease in human and animal, characterized by circulating antibodies against the desmosomal cadherin desmoglein 3 (Dsg3). We utilized a PV mouse model, in which a function disrupting mouse monoclonal anti-Dsg3 antibody (AK23) targets desmosomal adhesion, which results in blister formation

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by interfering with intracellular signaling events. Upon AK23 injection into eight-week-old C57Bl/6J mice, adhesion of telogen HF bulge SC is disrupted between the inner K6pos bulge layer, believed to maintain quiescence, and the outer CD34pos cell layer, which participates in HF renewal. Timecourse studies revealed that 24 hours after AK23 injection a prominent SC activation is induced. This is characterized by progressive loss of label retaining cells in K5-tTA;tetO-H2BGFP mice and increased SC proliferation. Concomitant downregulation of the quiescence marker NFATc1, reduced CD34 expression and downregulation of the bulge "stemness signature" (Sox9, Lhx2, Tcf4, Lgr5) further indicated profound alterations in SC homeostasis. We uncovered that BMP2/4 mRNA levels started to decrease from 24 hours onwards, correlating with PI3K/Akt activation. *In vivo* experiments using a dual PI3K/mTOR inhibitor (NVP-BEZ235) identified PI3K/Akt signaling to be involved in the late proliferative process, but early proliferation taking place around 24 hours after AK23 injection occurs prior to PI3K/Akt activation. Furthermore, PI3K/Akt signaling is not involved in loss of the "stemness signature." Instead, we discovered that Wnt-mediated Shh activation which usually parallels reduced BMP signaling and is a prerequisite for HF regeneration, is suppressed upon disruption of niche adhesion. We propose that these collective processes favor niche repair at the expense of HF regeneration. Indeed, SC are not lost in the long-term, although clonal efficiency is transiently reduced. Keratinocytes isolated from AK23 treated CAG-EGFP mice are able to replenish all skin lineages in single cell graftings. This suggests that SC activation did not result in irreversible commitment which is underscored by studies using K1-15-CrePR1;R26R mice to trace bulge-derived SC. No significant upward migration of activated bulge SC was observed upon disruption of niche adhesion. Furthermore, AK23 injected mice are able to enter anagen upon hair plucking. In summary we demonstrate in a mouse model for PV, that Dsg3 holds an important function in SC homeostasis by safeguarding quiescence. We further identify BMP/Wnt/Shh signaling as a switch board governing niche repair by receiving signals from early effectors upon disruption of niche adhesion. Therefore our work provides novel insights into cadherin-mediated SC activation and repair mechanisms.

Poster Board Number: 1148

NOTCH SIGNALING IN EPIDERMAL AND HAIR CYCLE HOMEOSTASIS

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Notch signaling regulates a variety of processes such as differentiation, proliferation, apoptosis, and cell fate choice. Ligand binding leads to conformational changes in the Notch receptor and leads to NICD release from the membrane. NICD translocates into the nucleus and binds to Rbpj and Mastermind, thereby activating downstream target genes. Protein O-fucose transferase-1 (Pofut1), which catalyzes O-fucose modification of Notch receptor extracellular domain, is required for efficient ligand-receptor binding and subsequent signal transduction. Although Notch signaling plays an important role in regulating early-stage differentiation of the epidermis and complete maturation of hair follicles, how Notch signaling participates in epidermal and postnatal hair cycle homeostasis is less studied. Here, we conditionally inactivated Notch signaling by deletion of Rbpj and Pofut1 using Tgfb3-Cre which induces gene recombination in hair follicle lineages and suprabasal layer of the epidermis. Both Rbpj and Pofut1 conditional inactivation resulted in defects in the granular layer and reactive epidermal hyperplasia. We are currently investigating gene expression levels of proteases and protease inhibitors which involved in filaggrin processing in the mutant epidermis. Disruption of Pofut in hair follicle lineages resulted in aberrant telogen morphology, a delay in anagen re-entry, and dysregulation of proliferation and apoptosis during the hair cycle transition. Moreover, increased DNA double stand break signals were detected in Pofut1-deficent hair follicles. To investigate the mechanism underlying the DNA damage response in Pofut1-deficient hair follicles, bulge stem cells from control and Pofut1-deficient mice were isolated and their colony forming ability was compared. Furthermore, examination of cell proliferation by BrdU staining and DNA damage by γ-H2AX staining were also carried out and results were compared between control and Pofut1-defeicint bulge stem cells. Our data suggest that Notch signaling plays a role in late-stage epidermal differentiation and postnatal hair cycle homeostasis.

Poster Board Number: 1150

THE BULGE AREA OF THE HAIR FOLLICLE IS THE MAJOR SOURCE OF HAIR FOLLICLE PLURIPOTENT STEM CELLS

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Nestin-expressing cells of both the bulge area (BA) and dermal papilla (DP) have been previously shown to be multipotent and be able to form hair follicle cells as well as neural and other non-follicle cell types. The objective of the present study was to determine the major source of nestin-expressing stem cells in the hair follicle and to compare the ability of the nestin-expressing stem cells from the BA and DP to repair spinal cord injury. Transgenic mice in which the nestin promoter drives GFP (ND-GFP) were used in order to observe nestin expression in the BA and DP. The expression of nestin in the DP depended on the phase of the hair follicle. Nestin-expressing DP cells were found in early and middle anagen. The BA had nestin expression throughout the hair cycle and to a greater extent than the DP. Nestin-expressing cells from both regions had essentially identical morphology with very long processes extending from them as shown by two-photon confocal microscopy. Nestin-expressing cells from both areas differentiated into neuronal cells at high frequency *in vitro*. Matrices of cells from both the BA and DP were formed by culturing them on Gelfoam® which were used to repair the injured spinal cord in nude mice. Both nestin-expressing DP and BA cells differentiated into neuronal and glial cells after transplantation to the injured spinal cord and enhanced injury repair and locomotor recovery within four weeks with the BA being the greater and more constant source.

Poster Board Number: 1152

DYNAMICS BETWEEN STEM CELLS, NICHE AND PROGENY IN THE HAIR FOLLICLE

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Here, we exploit the hair follicle to define the point at which stem cells become irreversibly committed along a differentiation lineage. Employing histone and nucleotide double-pulse-chase and lineage tracing, we show that the early SC descendents en route to becoming transit-amplifying cells retain stemness and slow-cycling properties and home back to the bulge niche when hair growth stops. These become the primary SCs for the next hair cycle, while initial bulge SCs become reserves for injury. Proliferating descendents further en route irreversibly lose their stemness, although they retain many SC markers and survive, unlike their transit-amplifying progeny. Remarkably, these progeny also home back to the bulge. Combining purification and gene expression analysis with differential ablation and functional

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experiments, we define critical functions for these non-SC niche residents, and unveil the intriguing concept that an irreversibly committed cell in an SC lineage can become an essential contributor to the niche microenvironment.

Poster Board Number: 1154

LHX2 OPERATES AS A SWITCHBOARD REGULATOR OF STEM CELL ACTIVITY DURING SKIN RESPONSE TO INJURY

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The Lhx2 transcription factor plays essential roles in the morphogenesis and patterning of ectodermal derivatives, as well as in controlling the activity of epithelial stem cells in the skin. We demonstrate here that in mouse and human hair follicles (HFs), Lhx2+ cells residing in the stem cell-enriched epithelial HF compartments (bulge, secondary hair germ, infundibulum/outer root sheath) represent the vast majority of cells that proliferate in response to skin injury. In heterozygous Lhx2 knockout (+/-) mice, the wound healing process is significantly retarded, while anagen onset in the HFs located closely to the wound edge is accelerated compared to wild-type mice. In Lhx2 -/- mice, expression of the genes encoding selected stem cell markers (Sox9, Tcf4, Cd34) was decreased, while expression of Lgr5 increased versus wild-type controls. Lhx2 co-localized with Sox9, Tcf4 and Cd34 in the bulge keratinocytes, and the number of Sox9+, Tcf4+ and Cd34+ cells seen in the infundibulum/bulge of the HFs closely adjacent to the wound decreased in the Lhx2 +/- mice versus wild-type controls. Furthermore, Chip-on-chip and Chip-qPCR analyses using lysates from primary mouse keratinocytes revealed Sox9, Tcf4 and Lgr5 as direct Lhx2 targets. Thus, our data strongly suggest that Lhx2 promotes re-epithelization during wound healing via positive regulation of Sox9 and Tcf4, while it simultaneously inhibits HF cycling via negative regulation of Lgr5. Thus, Lhx2 operates as a switchboard regulator of epithelial stem cell activity in the HF during the response of the skin to injury.

INTESTINAL/GUT CELLS

Poster Board Number: 1156

DIRECTED DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS INTO PROXIMAL INTESTINAL TISSUE

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Human pluripotent stem cells (PSCs), including embryonic stem cells and induced pluripotent stem cells, have a well-established capacity for differentiating into tissues derived from all three germ layers. In the endoderm, advancements in the study of foregut and hindgut organogenesis have permitted the establishment of protocols for the directed differentiation of PSCs into specific endoderm organ cell types. Recently, an *in vitro* culture system was described by our group in which three-dimensional intestinal tissue is robustly derived from PSCs. In this system, PSCs are differentiated into definitive endoderm (DE) with ActivinA, after which WNT3A and FGF4 induce posterior endoderm patterning and hindgut morphogenesis by 48 to 96 hours. Intestinal organoids emerge from culture, are able to be expanded in pro-intestinal conditions, and consist of mesenchyme surrounding polarized intestinal epithelium with specified intestinal cell types including Paneth, goblet, enteroendocrine, and LGR5+ cells. Although this tissue is clearly intestinal, we have not yet determined the proximal-distal nature of these organoids. Based upon the presence of crypt-like proliferative zones and villus-like structures within mature organoids, we hypothesized that they are proximal intestine. To investigate this we have analyzed tissue from our WNT/FGF cultures using proximal and distal intestinal markers. Expression of the distal small intestine marker GATA6 was absent and expression of both GATA4 and KLF5, known to be present in developing proximal intestine, was increased nearly 4-fold compared to control cells exposed to no growth factors. Additionally, emerging organoids express increased levels of HoxC5 and HoxC9 compared to controls. To investigate if we could alter the proximal-distal fate of intestinal tissue we included the factor BMP2 to the WNT3A/FGF4 condition. BMP signaling is known to be critical to intestinal morphogenesis and thought to play a role in proximal-distal development of the gut. Organoid production still occurred but was decreased by 80% in the presence of BMP2. Whereas expression of GATA4, GATA6, and KLF5 was absent, expression of the distal intestinal markers HoxA13 and HoxD13 was significantly increased with addition of BMP2 compared to both control and WNT/FGF alone. We also analyzed for the presence of specific enteroendocrine cells, which are normally present in discrete, proximal-distal segments of the gut. Intestinal organoids cultured long-term for 4 months were positive for chromograninA and the duodenal region-specific hormones glucose-dependent insulinotropic peptide (GIP) and cholecystokinin. However, they were negative for glucagon-like peptide 1 (GLP1), ghrelin, peptide YY (PYY) and somatostatin. Taken together these data suggest that our culture conditions promote a proximal hindgut fate as indicated by region-specific gene expression, and mature intestinal organoids are similar to fetal duodenal tissue by morphology and region-specific enteroendocrine hormone expression. Furthermore, addition of BMP2 may induce a more posterior gut pattern compared to WNT3A and FGF4 alone. Ongoing studies are aimed at further characterization of the regulation of patterning in organoid development and at factors capable of inducing more proximal or more distal intestinal tissue.

Poster Board Number: 1158

PREGNANCY ASSOCIATED PROGENITOR CELLS ENGRAFT IN THE MATERNAL MURINE SMALL INTESTINE AND DISPLAY A HEMATOPOIETIC PHENOTYPE

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Cells of fetal origin known as Pregnancy Associated Progenitor Cells (PAPCs) are known to migrate to the mother during parturition and survive for long term. This phenomenon of fetal cell microchimerism has been widely studied in the mammalian system and PAPCs are shown to exhibit multilineage potential. Clusters of PAPCs were reported in the studies of human small intestine and we sought to characterize intestinal PAPCs in a murine model in more detail. We studied the presence of GFP labeled PAPCs in the maternal intestine pre- and postnatally. PAPCs were detected in the intestine in 42-60% of mothers; earliest at postpartum day 10 with long term engraftment up to 7 months postpartum. PAPCs were located in the lamina propria and at the periphery of the Peyer's patches in a non-specific manner throughout the entire length of the small intestine. PAPCs did not proliferate in the intestine and were identified to be of hematopoietic nature as judged by the expression of B and T lymphoid specific markers such as CD19, IgA,

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CD4, and CD8 and the myeloid marker, CD11b. PAPCs expressing the hematopoietic markers CD34+ and CD45+ were also found in the maternal peripheral blood at timepoint P0 and P40 thus suggesting the presence of a long term hematopoietic progenitor pool in mothers. A subset of PAPCs in the maternal peripheral blood as well as in the small intestine expresses α4β7 integrin which is involved in specific homing to intestinal lymphoid tissues and other major lymphoid organs that express mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Our current research focuses on identifying the mechanisms of PAPC migration to the mother and to specific organs such as the small intestine.

Poster Board Number: 1160

THE ROLE OF β-CATENIN AND EOMESODERMIN IN THE ESTABLISHMENT OF MOUSE PROGENITOR AND STEM CELL LINEAGES DURING INTESTINAL ENDODERMAL DEVELOPMENT

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Many disorders, including various types of cancer, are a direct result of the impaired development of the primitive gut tube, which is derived from the endodermal germ layer. Although the canonical Wnt pathway is central for many developmental processes, few studies have examined Wnt and its key player, β-catenin, in early endoderm specification. Most of the present insight comes from adult epithelial studies, which poses severe limitations to the understanding of gastrointestinal pathologies. Similarly, Eomesodermin, a crucial gene in trophectoderm development, has only recently been implicated in definitive endoderm specification. Importantly, many of the genes that, in articulation with Eomesodermin, orchestrate trophectoderm lineage establishment in the early embryo, including Nodal, Cdx2, Fgf4 and Ascl2, will later prove decisive in initiating posterior endodermal fates and intestinal identity. Furthermore, an interaction between β-catenin and Eomesodermin was reported in progenitor populations of the developing brain cortex. However, the extent and nature of this interaction during the establishment of the intestinal lineage remain unknown. In order to determine the role of β-catenin and Eomesodermin in the regional specification of gut endoderm progenitors, we designed a novel approach combining Cre-mediated mutagenesis and experimental explants. To overcome the embryonic lethality of both β-catenin and Eomesodermin mutant embryos, we crossed mice with conditional (floxed) β-catenin stabilized, β-catenin null and Eomesodermin null alleles, with the inducible Cre-driver mouse line, Claudin-6CreERT2, which is specific to the endodermal compartment. Also, by tuning the tamoxifen dosage, mosaic recombination will be enabled by the CreERT2 system, and used for clonal studies. At present, we are conducting an exhaustive phenotypical analysis, employing several techniques including antibody staining, confocal imaging and whole-mount *in situ* hybridization, in conjunction with biochemical studies and the establishment of *in vitro* organoid cultures from mutant and control mice. The present study will be instrumental in disclosing the transcription factor networks and mechanisms involved in endoderm and intestinal progenitor establishment. This knowledge is absolutely critical to a better understanding of the ontogeny of various gut diseases, and will encourage the development of novel therapeutics, considerably improving human health.

Poster Board Number: 1162

THE MAMMALIAN INTESTINE CONTAINS SEPARATE NEURAL CREST AND PANCREAS DERIVED STEM CELLS

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Adult stem cells are found amongst differentiated cells in a tissue or organ; they are capable of self-renewal and are thought to be limited to produce only the differentiated cell types of their tissue of origin. Currently, two types of stem cells are identified in the adult mouse gut: first, Lgr5 positive crypt based gut stem cells that generate all epithelial lineages, and second, enteric neural crest stem cells (NCSC) that persist in the gut muscle and can be cultured as clonal spheres and give rise to neurons, glia and smooth muscle cells among others. Here we report clonal isolation of sphere forming cells from the external muscle layer (EML) of the adult small intestine. Most of these clonal spheres co-expressed a combination of NCSC markers and generated differentiated neural crest progeny. Surprisingly, a smaller, separate population of clonal spheres produced pancreatic cell type progeny upon differentiation. Using Pdx1 (an early marker of the pancreatic and duodenal lineages) Cre x Roza Yfp transgenic mice we observed the clonal formation of Yfp -expressing spheres. The differentiation of FACS-sorted Yfp+ (Pdx1 lineage derived) precursor derived spheres revealed that the progeny of these spheres had became a variety of differentiated pancreatic cell types expressing endocrine (Pdx-1, c-peptide) and exocrine-(amylase) markers as well as a neuronal cell type marker (b3-tubulin+ cells), whereas clonal spheres derived from Yfp- cells did not give rise to any pancreatic progeny. Immunolabeling of a whole adult duodenum and ileum tissue indicated the existence of the Yfp-positive (Pdx1 lineage derived) network, corresponding to portions of the enteric nervous system. Additionally, whole duodenal and ileal tissue was co-stained for Map2 (a marker of neuronal cells) and Yfp protein using immunofluorescent techniques. Co-expression of the both markers was found in a minority of the Yfp+ cells of the enteric plexus (and thus are Pdx1-derived neurons), and the majority of cells expressed only Map2 (and presumably are neural crest derived neurons). Some of the cells in the EML of duodenum and ileum expressed only Yfp, and may be the Pdx1-derived stem cells that formed clonal spheres *in vitro*. Analysis of early transgenic embryos revealed that Pdx1 progeny migrate from developing pancreas and duodenum at E11.5 and contribute to enteric neural system. These results provide evidence that the enteric nervous system arises form both the neural crest and the endoderm. In the adult animals both neural crest and pancreatic stem cells are present in the external muscle layer of the gut. Identification of progenitor cells derived from gut adult tissue as a novel intrinsic source of pancreatic precursor cells has implications as potential expandable source for replacement therapy for diabetes.

Poster Board Number: 1164

REGULATION OF INTESTINAL REGENERATION BY INSULIN/NOTCH SIGNALING INTERACTIONS

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Pluripotent intestinal stem cells (ISCs) are critical for the maintenance of the adult posterior midgut. ISC proliferation is dynamically regulated in response to various environmental challenges and can be controlled by signals emanating from surrounding enterocytes (ECs) and/or visceral muscle cells, as well as by systemic growth regulators such as insulin-like peptides. ISCs divide asymmetrically, producing a new stem cell and a partially differentiated enteroblast (EB). Notch activation in EBs promotes their differentiation into either the absorptive ECs or into secretory cells known as enteroendocrine cell (EEs). While insulin/IGF signaling (IIS) activity in ISCs promotes their pro-

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liferation, we find that the same pathway also regulates endoreplication and growth of differentiating EBs, and influences lipid and glycogen metabolism in terminally differentiated ECs. The cellular response to IIS activation thus changes in the ISC lineage as cells progress toward terminal differentiation. How these different responses to IIS activation are achieved, and how the response of ISCs, EB and ECs changes during the commitment and differentiation process, remains unexplored. Our preliminary results suggest a critical role for the Tuberous Sclerosis Complex (TSC) in diversifying the response of the ISC lineage to insulin signaling. TSC is an inhibitory component of TOR signaling, and TOR is required downstream of the insulin receptor to promote cell growth. Our results suggest that Tor signaling regulates growth of EBs, but does not influence proliferation of ISCs. We observe high levels of TSC2 expression in ISCs, and a transient reduction of TSC2 in EBs during the commitment and differentiation process, suggesting that the differential expression of this protein determines the specific response of ISCs and EBs to IIS activation. Our data further indicate that this transient repression of TSC2 expression is caused by elevated Notch signaling activity in EBs. The molecular mechanism of this interaction is currently under investigation.

EPITHELIAL CELLS (NOT SKIN)

Poster Board Number: 1166

CD93 AND CD31 AS POSSIBLE CANDIDATE MARKERS FOR HUMAN ENDOMETRIAL STEM/ PROGENITOR CELLS

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The human uterine endometrium undergoes cyclical regeneration throughout a woman's reproductive life. Its high regenerative capacity suggests the existence of endometrial stem/progenitor cells. Recently several endometrial cell subpopulations have been emerged as candidate endometrial stem/ progenitor cells including clonogenic endometrial cells and endometrial side population cells (ESP) which possess a Hoechst 33342 low-fluorescence profile. In particular, several groups including us have demonstrated that human ESP, but not endometrial main population cells (EMP), exhibit stem cell-like properties including self-renewal, dormancy, and potential for differentiation into a variety of endometrial cell components and that at least some ESP are localized to endometrial vascular endothelial walls. However, side population-based cell isolation is limited in terms of extreme expensiveness of keeping and using ultraviolet laser-equipped flow cytometry, cell damage caused by UV exposure, and toxicity of Hoechst dye. Therefore, to facilitate possible future clinical use, identification of surface markers is needed to permit selection of the endometrial stem cell population. The objective of this study was to search for genes associated with the surface phenotype and stem cell function of ESP. For this purpose, we isolated ESP and EMP from human endometria and compared the gene expression profiles between ESP and EMP using HG-U133A&B Affymetrix GeneChip platform. Microarray analysis revealed that 117 genes (143 probe sets) and 40 genes (51 probe sets) were up-regulated and down-regulated in ESP, respectively, as compared to those in EMP. The up-regulated genes of ESP contained many vascular endothelial-associated genes including CD31, vascular endothelial growth factor receptor 2, CD34, thrombomodulin, vascular endothelial-cadherin, and CD93. In addition, ESP preferentially expressed transcription factors such as hematopoietically-expressed homeobox protein and GATA-binding protein 3. The up-regulation of these genes was further confirmed by qPCR. We focused on CD 93 and CD31, because CD93 distinguishes a rare human stem cell population with both hematopoietic and hepatic potential, and also CD31+ESP still show stem cell-like properties (unpublished observation). We performed immunohistochemical studies using human endometrium and found that CD93+CD31+cells were preferentially localized to endothelial walls, whose distribution was similar to that of ESP. These results implicate CD93 and CD31 as possible candidate markers for human endometrial stem/progenitor cells.

Poster Board Number: 1168

HUMAN SOLID ORGAN CANCERS CONTAIN STEM-LIKE LABEL RETAINING CANCER CELLS THAT UNDERGO ASYMMETRIC CELL DIVISION AND HAVE SUPERIOR TUMOR INITIATING CAPACITY

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Label-retaining cells (LRC) were identified in several tissue types and have been proposed to represent adult tissue stem cells. Cancers develop in all tissue types where LRC were detected. LRC are hypothesized to result from either slow-cycling or asymmetric-cell-division with non-random chromosomal cosegregation (ACD-NRCC). However, the stem-cell nature and whether LRC undergo ACD-NRCC remains highly controversial. LRC are identified by exposing cells during development to nucleotide analogues such as Bromodeoxyuridine (BrdU) or 3H-thymine-deoxyribose (3H-TdR). During a chase period, where nucleotide analogous are no longer available to the cells, the DNA labels are diluted with each subsequent cell division, and theoretically will become undetectable after 4-5 cell divisions. Surprisingly, several investigators found that there is a subpopulation of cells that retain the DNA labels over a prolonged period of time. These cells are called LRC. In several studies, LRC were associated with populations of cells comprised or highly enriched with adult tissue stem cells. However, other investigators questioned the stem cell nature of LRC. The mechanism by which LRC are generated continues to be debated. It has been proposed that LRC are the result of either relative quiescence/slow-cycling or ACD-NRCC. The major obstacle for testing LRC and these hypotheses is that currently detection of LRC is done on fixed tissue. Here we show that we developed a unique method allowing isolating live LRC. This method allowed us, for the first time, to test the stem cell nature of LRC and the hypotheses regarding their mode of generation. Using this novel method for isolation of live label-retaining-cancer-cells (LRCC), we provide evidence that a subpopulation of LRCC is not quiescent, actively dividing (10-16% in active mitosis), and express stem-cells and pluripotency associated genes. Using fluorescence-labeled nucleotide analog and real-time confocal-microscopy cinematography, we show directly that LRCC undergo live asymmetric non-random chromosomal cosegregation label-retaining cell division for the first time. Using gene expression analysis we propose pathway-map containing driver genes of LRCC. Finally, in large blinded study, we show that LRCC have greater tumor initiating capacity than non-LRCC in-vivo. Additionally, we show that liver cancer derived LRCC are resistant to Nexavar. Based on our data and the fact that LRC are ubiquitous, LRCC might represent a novel population of universal stem-like cancer cells. LRCC has the potential to provide innovative targets for the treatment of cancer and novel mechanistic insight into the biology of cancer.

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Poster Board Number: 1170

P63 PROMOTES QUIESCENCE IN HUMAN AND MOUSE MAMMARY STEM CELLS VIA GLOBAL SUPPRESSION OF RNA POLYMERASE II ACTIVITY AND SELECTIVE EXPRESSION OF ANTI-PROLIFERATIVE P53 TARGETS

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Mammary stem cells survive mammary gland involution and permit cyclical epithelial regeneration. Recently it was reported that a variety of adult stem cells display diminished phosphorylation at Serine 2 of the RNA Polymerase II carboxy-terminal domain, a post-translation modification associated with productive mRNA elongation. We had observed changes in expression levels of so-called housekeeping genes in models of quiescent mammary stem cells, suggesting a role for the suppression of Pol II activity. Analysis of RNA Pol II in a hTERT immortalized model of human mammary stem cells revealed sharp declines in the phosphorylation at S2 at time points corresponding to reversible cell cycle arrest. Both the reduced S2 phosphorylation and cell cycle exit are P53-independent. ChIP analysis demonstrated stalled polymerases as a functional consequence of reduced CTD phosphorylation. In an immortalized mouse mammary progenitor-like cell line, ectopic expression of ΔNp63α, the predominant TP63 isoform in mammary stem cells, induced a quiescent state characterized by prolonged G1, reduced S2 phosphorylation, and reduced total RNA levels. Additionally, we observed that both spontaneous quiescence and quiescence induced by ectopic ΔNp63α were characterized by enhanced expression of anti-proliferative P53 target genes. These findings suggest a mechanism enabling stem cell resistance to differentiation and apoptosis by blocking elongation of a large subset of genes. To what degree human breast cancer stem cells share the features of quiescence is an open question with important implications for targeted therapies.

Poster Board Number: 1172

CHARACTERIZATION OF EPITHELIAL STEM CELLS IN THE MOUSE INCISOR USING GENE EXPRESSION PROFILING

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Rodents have a highly specialized dentition with incisors that grow continuously due to the presence of adult stem cells at their proximal end. These stem cells ensure generation of all required differentiated cell types, both epithelial and mesenchymal, throughout the animal's life. The mouse incisor provides a good model system to study stem cells for two reasons. First, the generation of distinct cell lineages from adult stem cells in the incisor appears to be controlled by mechanisms shared with several other systems. Second, owing to its unidirectional growth, the progeny at progressively increasing stages of maturity are arrayed in a linear fashion, such that stage-specific effects of genetic and biochemical perturbations are readily identifiable. Using a combination of antibody staining, lineage tracing and pharmacological approaches, we previously showed that a subset of the epithelial stem cells in the mouse incisor is Gli1-positive and requires Hedgehog (Hh) signaling for proper generation of progeny. Whereas these stem cells give rise to multiple epithelial cell lineages, Hh signaling is required specifically for generation of only one of the cell types, the enamel-producing ameloblasts. In the studies reported here, we performed microarray analysis to obtain genome-wide expression profiles of the stem cell-containing proximal incisor region. Samples were obtained from wild-type mice treated with either a small molecule Hh-Antagonist or a biologically inactive control. The most differentially expressed genes between these two groups (fold change greater than 2, false discovery rate less than 0.1) were identified as candidate genes downstream of Hh signaling that potentially could be involved in regulation of lineage-specific progenitors. First, we selected those differentially expressed genes that had been reported to be expressed in stem cells or the niche in other systems, and we are studying the spatial expression patterns of those genes in the incisor in more detail. Second, using a more holistic approach, we also analyzed gene co-expression network organization in the microarray data. This approach revealed a number of large gene co-expression modules that are likely to relate to distinct biological processes that are active in this region during development. Previous work has shown that gene co-expression modules identified in heterogeneous tissues are often driven by discrete cell types; therefore, we are currently investigating whether identified modules are enriched with known markers of incisor cell types. The identification of such modules will provide a wealth of new biomarker information and enhance our understanding of the cellular diversity that is present in this region. Together, these experiments are enabling us to delve deeper into the molecular basis for generation of cell type specific progenitors from the Hh-responsive stem cells by delineating the signaling pathways involved in this process. Additionally, these studies should point us to novel markers that allow discrimination between different cell types in the incisor stem cell niche. The results of these studies will facilitate our studies of adult stem cells and will further our understanding of how lineage decisions are directed.

Poster Board Number: 1174

OVARIAN GRANULOSA CELLS: A NOVEL CELL SOURCE WITH OSTEO-REGENERATIVE POTENTIAL

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Granulosa cells (GC) have recently been shown to express stemness markers and be capable of differentiating into cell types not present within the follicles. Since ovarian follicles are in continuous dynamic evolution we have undertaken this research in the pig model to identify the phase of follicular dynamics from which GC with the best stemness properties and highest regenerative potential can be retrieved. Prepubertal gilts were treated with 1200 IU equine chorionic gonadotropin (eCG) to induce follicle growth followed 60 h later by 500 IU human chorionic gonadotropin (hCG) to trigger follicle luteinisation eventually leading to ovulation in 40h. GC from growing follicles (GGC) were isolated from the ovaries of gilts ovariectomized 50h after eCG, while GC from luteinizing follicles (LGC) were obtained from ovaries removed 30h after hCG. GC were expanded *in vitro* for 3 successive passages in αMEM based growth medium. Cell plasticity was assessed by culturing GC for 4 weeks in osteogenic medium (αMEM supplemented with 50 μM ascorbic acid, 10 mM β-glicerol phosphate, 0.2 μM dexamethasone). Stemness markers (Nanog, SOx2 and TERT) GC markers (FSH receptors, inhibin, estradiol and progesterone production) were evaluated immediately after cell isolation, at the end of the expansion *in vitro* and after osteogenic differentiation. Osteogenic differentiation *in vitro* was assessed by evaluating extracellular matrix mineralisation (Calcein deposition, Alizarin red staining) and the expression of bone related genes (osteocalcin, Runx2). Cultured on poly(DL-lactide-co-glycolide) (PLGA) scaffolds for 2 weeks in osteogenic medium, GG, traceable by the membrane dye PKH26, were implanted s.c. in the dorsal region of SCID mice to confirm their osteo-regenerative potential *in vivo*. Immediately after isolation both GGC and LGC cells showed a diffused expression of Sox2, Nanog and TERT. Expansion *in vitro* caused

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in both cell types a progressive disappearance of granulosa cell characters; expression of FSH receptors was barely detectable after three passages, steroid production dropped to 1/10 of the initial levels and inhibin positive cells were totally absent at the end of the expansion. By contrast, expansion did not substantially modify the expression of stemness markers both in GGC and LGC. Extracellular matrix mineralisation was clearly recorded in LGC cultured for two weeks in osteogenic medium, while it was totally absent in the same cells cultured in growth medium. Matrix mineralisation was much lighter in GGC, becoming clearly detectable only after 4 weeks of culture. RT-PCR showed that osteocalcin and Runx2 expression were upregulated while stemness markers were down regulated by osteogenic medium in both cell types although LGC underwent wider changes. Implants retrieved 8 weeks after transplantation showed viable GC surrounding the several nodules of calcifications recorded. Similar osteogenic effect was induced by GGC and LGC while no bone formation was observed in the control scaffolds without cells. These data confirm the stemness properties of GC, show that *in vitro* expansion cause a progressive de-differentiation of granulosa cells without affecting their staminality and demonstrate that both GGC and LGC have ostegenic potential, luteinizing cells being the more efficient. Transplanted in SCID mice GC can directly participate in new bone formation thus confirming their therapeutic potential.

Poster Board Number: 1176

SUPPRESSION OF THE IMMUNE SYSTEM BY OVARIAN CANCER CELLS

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Ovarian cancer is a devastating disease with only 30% 5-year survival despite cytotherapy, extensive surgery and Paclitaxel treatment. Reoccurrence of the disease is in most cases again in the abdomen. Recurrence of disease has been explained by the resistance of cancer stem cells to standard therapies. Ovarian cancer creates an immune suppressive microenvironment by recruiting Tregulatory cells from the circulation. This immune suppression appeared to be more extended than anticipated. Lymphocytes collected from the ascites were non responsive to full activation signals in several patients. Removal of suppressive Tregulatory cells and myeloid derived suppressor cells by flow-sorting did not improve the reactivity of the ascites derived lymphocytes. Ascites fluid itself was able to suppress lymphocyte reactivity. Lymphocytes obtained from the blood of the same patient were normally responsive. We assume that circulating responsive lymphocytes migrate into the abdomen and become non-responsive in time. This opens the possibility to induce an immune response to cancer stem cells after treatment. Immune therapy directed to cancer stem cells could add to the armamentarium to fight this tumor. Sphere like clusters of tumor cells were observed when tumor cells were cultured under floating conditions in serumfree medium as was used for other epithelial cancers. Therefore, m-RNA of ovarian cancer stem cells was transfected into dendritic cells and experiments are under way to determine DC induced immunity. Transfected dendritic cells matured normally after incubation with maturation cytokines. Presently, we investigate if transfected DCs are able to activate a CTL response to ovarian cancer stem cells.

EYE OR RETINAL CELLS

Poster Board Number: 1178

ERG EVALUATION OF RETINAL FUNCTION AFTER STEM CELL TRANSPLANTATION

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Purpose: To determine if human retinal progenitor cells (hRPCs) can improve retinal function upon transplantation in an animal model of retinal degeneration (C3H SCID mice) and to determine the effect of different oxygen concentrations on the hRPCs in terms of growth and functional outcome. Methods: hRPCs were grown under high oxygen (20%) and low oxygen (3%) conditions. Characterization of cells was done at specific points (P2, P6, P10). hRPCs were transplanted into the subretinal space of C3H SCID mice, then ERG was performed after 3 weeks. Animals were fixed on a specially designed heated stage, ERG contact lens electrodes were placed on the cornea, and mice were moved into a Ganzfeld stimulator. ERG responses of the treated eyes were compared with the untreated eyes as a control. Animals were then euthanized and immunohistochemical staining was done to assess the morphology of the transplanted cells *in vivo*. Results: ERG recordings showed a statistically significant improvement in the treated eyes compared to the untreated eyes. This improvement could be traced after injection of different passages of cells up to P15. Although Low oxygen condition (3%) was found to promote hRPCs expansion, it had no significant effect on ERG improvement. Immunohistochemical studies of treated eyes showed that hRPCs had the ability to differentiate within the host tissues and to express some photoreceptor markers as CRX, Recoverin and Cone Opsin. Some cells showed evidence of integration through expression of Synaptophysin, which is an indicator of synapse function. Conclusion: hRPCs transplantation could be a potential tool for improving retinal function in the setting of retinal degenerative disorders. We can also conclude that using low oxygen concentration in culture promotes hRPCs expansion.

Poster Board Number: 1180

ADULT HUMAN RPE CAN BE ACTIVATED INTO A MULTIPOTENT STEM CELL THAT PRODUCES RPE AND MESENCHYMAL DERIVATIVES

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The retinal pigment epithelium (RPE) forms a single layer of highly specialized cells performing many functions needed for a functioning neural retina. In normal conditions, RPE remain non-proliferative throughout adult life. In lower vertebrates during retinal injury, RPE cells can re-enter the mitotic phase and produce progeny capable of regenerating all cells of the retina and in some cases also the lens. Retinal stem cells, as defined by their ability to self-renew *in vitro* and differentiate into retinal neurons and glia, have been identified in the ciliary epithelium and iris pigmented epithelium of adult rodents and human. However, until now, there has been no evidence for a stem-like cell from the adult RPE. We hypothesized that similar to its corresponding lower vertebrate equivalent, the adult human RPE retains dormant properties of early neuroepithelial cells that can be activated if removed from its normal environment. To test our hypothesis, we isolated RPE cells from adult human cadaver eyes. When cultured *in vitro*, RPE cells exhibit two characteristic morphologies: epithelial pigmented "cobblestone" cells or fusiform cells with low pigmentation. These cells proliferate actively and self-renew over many passages, and they express markers associated with the stem cell state, including SSEA-4, c-Myc and KLF4. To determine

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their plasticity, both RPE culture types were examined for their potential to form neural progeny as well as mesoderm and endoderm by inductive treatments for neural, osteogenic, chondrogenic, myogenic, adipogenic and hepatic lineages. We found that under appropriate culture conditions RPE cells are highly proliferative and are able to differentiate into an unexpectedly wide repertoire of progeny, including bone, cartilage, muscle and adipocytes, but not endoderm. To rigorously prove multipotency, we performed clonal experiments in which single primary RPE cells were clonally expanded then split into five differentiation conditions; such clonal analyses showed that RPE clones can differentiate into multiple lineages. This multipotency observed in human RPE can be generalized across mammalian species, as RPE derived from cow eyes showed the same capabilities. This study establishes the human RPE to be a unique source of multipotent stem cells, RPESCs that are distinct from neural stem cells. Moreover, these cells can be obtained from patients, offering the possibility of autologous transplantation therapy. Patient-matched stem cell lines derived from RPE biopsies could be a unique source of multipotent CNS-derived stem cells for the study of cell fate choice and could be used to generate specific cell types, including RPE, for autologous or allogeneic cell replacement therapy.

Poster Board Number: 1182

ADULT HUMAN CIRCULATING ANGIOGENIC CELLS (CACS) ARE NOT ENDOTHELIAL PROGENITOR CELLS (EPCS) BUT REPRESENT A TYPE OF M2 MACROPHAGE THAT FACILITATES ANGIOGENESIS THROUGH IL8

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EPCs promote angiogenesis and facilitate vascular repair. Recent clinical trials have demonstrated the feasibility, safety, and effectiveness of EPCs for treatment of various ischaemic diseases. However, results from such trials have been inconsistent. This is due to the heterogeneous mix of cells used as the term EPC has been applied to a broad range of blood-derived cells that display some endothelial markers and enhance angiogenesis. We have previously characterised two EPC subsets *in vitro*, CACs and Outgrowth endothelial cells (OECs). Although OECs are the only EPC subset with true progenitor and endothelial characteristics, CACs may still play an important role angiogenesis indirectly, as they have been shown to modulate vascular repair through the release of paracrine factors. The purpose of this study was to characterise the molecular phenotype of CACs, examine their role in angiogenesis, and elucidate molecular mechanisms responsible for CACs pro-angiogenic effects. Despite being widely referred to as EPCs, we reveal CACs to be cells lacking any endothelial characteristics; they do not directly incorporate into retinal microvascular tubes *in vitro* or a vascular network *in vivo*, however they significantly induced endothelial tube formation ($p<0.001$) and enhanced vascular repair in a murine model of ischaemic retinopathy ($p<0.001$) compared to respective controls. Our data highlights CAC-derived interleukin 8 (IL-8) as one of the key cytokines responsible for their pro-angiogenic function, as blocking IL-8 but not VEGF prevented CAC-induced angiogenesis. Furthermore we demonstrate that extracellular IL-8 is capable of transactivating VEGFR2 and induces the phosphorylation of ERKs in mature endothelial cells. Analysis of CACs transcriptome and immunophenotype suggests that they closely resemble M2 macrophages, as they express typical M2 markers such as CD163, CD204, CD206 and IL-10. Our findings unequivocally demonstrate a role for CACs in angiogenesis, highlight IL-8 as a key paracrine factor involved and reveal for the first time CACs to represent M2 macrophages with anti-inflammatory and pro-angiogenic properties.

Poster Board Number: 1184

CHARACTERIZATION OF HUMAN ORAL MUCOSAL EPITHELIAL CELLS FOR FUTURE USE AS AN ALTERNATIVE SOURCE OF AUTOLOGOUS STEM CELLS FOR THE TREATMENT OF OCULAR SURFACE DISEASE

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Introduction: Corneal structural integrity and transparency is necessary and critical for vision. The epithelium is the outermost layer of the cornea and its homeostasis relies on limbal epithelial stem cells (LESC) that are located at the basal layer of the limbus. LESC give rise to transit amplifying cells (TAC) in order to replenish suprabasal epithelium that is lost during normal eye blinking. Loss of LESC because of injury or disease results in cornea damage and can lead to blindness. Cultured LESC therapy can be used to treat LESC deficiency using autologous cells (from the healthy eye) or allogeneic donor cells. However, limited donor tissue supply, and importantly the need for systemic immunosuppression with the use of allogeneic LESCs, points to the need for an alternative solution. Oral mucosal epithelial cells (OMEC) have been used as an alternative cell source for treatment of LESC deficiency. However, post-operative follow up of the patients reveals susceptibility to undesirable vascularization of the peripheral cornea. Aim: To compare the characteristics of cultured human OMEC with those of limbal epithelial cells (LEC) in order to optimize OMEC culture conditions prior to grafting for the treatment of ocular surface failure. Methods: OMEC were isolated from 3mm punch biopsies of buccal mucosa from healthy volunteers. LEC were obtained from human cadaveric donor corneas. OMEC and LEC were cultured in presence of growth arrested 3T3 fibroblasts and fed with OMEC/LEC medium respectively every other day. For both cell types, nucleus/cytoplasm (N/C) ratio and colony forming efficiency assays were performed. Immunocytochemistry was used for investigation of ABCG2, CK12, CK19, p75, pax6 and p63alpha expression. Results: Small colonies of OMEC and LEC were observed within 5 days of isolation. Primary (P0) human OMEC had a significantly higher N/C ratio (0.3483 ± 0.01744) compared to that of primary LEC (0.2806 ± 0.01414) ($n=5$, ±SEM, $p< 0.05$, two-tailed t-test). There was no significant alteration of the mean N/C ratio across different passages of OMEC (P0, P1, P2). Colony forming efficiency assays showed no significant difference between the two cell populations. Immunocytochemistry revealed the expression of nuclear p63alpha, cytosolic / membranous ABCG2 and CK19 by both cell types. OMEC did not express p75 and pax6. CK12 and pax6 expression was apparent in LEC. Conclusion: Primary OMEC have higher N/C compared to LEC in primary culture, however CFE assays demonstrated no significant difference in the proliferative capacity between the two cell types. This demonstrates the possible similar proportion of stem cell progenitors among the two populations. OMEC remain relatively undifferentiated across passages which allows the manipulation and use of OMEC for longer periods, an advantage for therapeutic use. Both cell types express p63alpha and ABCG2, demonstrating the possible presence of progenitor cells in both populations. Overall, the present study demonstrated the phenotypic characteristic of OMEC and their differences to LEC. According to these results it is possible to use pax 6 as a marker for detecting possible transdifferentiation of OMEC into corneal epithelial cells when altering the culture conditions since pax6 is used as a marker for normal corneal epithelial differentiation.

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Poster Board Number: 1186

OPTIMIZATION OF RETINAL DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS BY MEANS OF MANIPULATING MICROENVIRONMENTAL OXYGEN TENSION

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Human pluripotent stem cells have the ability to differentiate into almost all types of cells in the body including retinal cells which raises hope for treating currently incurable ocular diseases such as Retinitis pigmentosa. Despite encouraging evidences of generating retinal cell types from human embryonic (hES) and induced pluripotent (iPS) stem cells, they all seem to suffer from one major pitfall; the low yield of differentiated cells. We sought to overcome the differentiation inefficiency by manipulating oxygen tension during differentiation protocol. Current differentiation protocols are performed at 20% O2 which is significantly higher than the O2 tension experienced by the developing mammalian (rabbit 8.7% and monkey 1.5%) and human embryo (~3%). In this study, we examined the effect of mimicking the physiological O2 tension on retinal differentiation of hES and iPS cells. Our results have shown upregulation of early eye field genes, Lhx2 and Six3 under lowered O2 compared to normal O2 after 3 days of differentiating cells as EBs in suspension. Upon attachment of EBs, the expression of retinal progenitor cell (RPC) markers, Pax6 and Chx10, as well as the early eye field genes was markedly increased when pluripotent stem cells were induced to differentiate under hypoxic condition. In addition, RPCs generated from both hES and iPS under normal and lowered O2 further differentiated into cells expressing Crx, a marker for photoreceptor precursor cells upon treatment of RA and taurine. These studies reveal the oxygen tension is a key element of the microenvironment during the retinal neural differentiation of pluripotent stem cells.

Poster Board Number: 1188

SPECIFICATION OF THE EARLY EYE FIELD FROM HUMAN EMBRYONIC STEM CELLS

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Several protocols have been published that efficiently drive human embryonic stem cells(hESC's) into retinal progenitors. These progenitors can be further differentiated into all cell types of the eye, including photoreceptors and retinal pigmented epithelium (RPE), but the precise molecular signaling pathways involved in ocular differentiation are poorly defined. To visualize early eye field differentiation from the pluripotent state, we have developed fluorescent reporter hESC lines under control of the Rax and Mitf promoters. Rax is expressed at the earliest stages of eye field specification. Mitf is first expressed throughout the early optic vesicle and is later restricted to the RPE, making it an ideal reporter for both early eye and RPE development. The most efficient protocol to induce ocular specification makes use of the soluble factors noggin, Dkk-1, IGF-1 and bFGF, giving rise to ~80% retinal progenitors as defined by Chx10 expression. From previous studies, it is known that these factors modulate BMP, Wnt, IGF and FGF signaling pathways. We have further characterized the roles of these pathways through loss of function experiments that utilize small molecule inhibition and RNA interference. Preliminary results show that the phosphoinositide 3-kinase (PI3K) pathway is necessary for initial neural induction prior to specification of the early eye field. These studies provide new insights into the mechanisms of early and late retinal differentiation from human embryonic stem cells, which may one day be used to treat degenerative ocular diseases such as age-related macular degeneration.

Poster Board Number: 1190

CHARACTERIZATION OF HUMAN LIMBAL STEM CELLS USING ACOUSTIC FOCUSING CYTOMETRY

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Limbal stem cells (LSCs) are found at the periphery of the cornea in the limbus and function to maintain and renew the corneal epithelium. *Ex vivo* expansion and transplantation of LSCs has been proposed as a treatment for various corneal diseases and injuries. In the present study we have developed a method to identify and characterize LSCs derived from primary human limbal tissue. Experimentation was focused on a model system composed of human corneal epithelial cells enzymatically released from normal corneal-scleral buttons and expanded in KSFM growth medium. Cells of various passage number were harvested and analyzed using multicolor acoustic-focusing cytometry. Previous work has demonstrated that stem cells specifically pump out the cell-permeant DNA-binding dye Vybrant® DyeCycle™ Violet via ABCG2-mediated efflux. This property enables the use of side-population technique to identify a sub-population of putative LSCs. The ABCG2 inhibitor Fumitremorgan C was shown to block the appearance of the corneal cell side-population. A549 human lung adenocarcinoma cells, which overexpress the ABCG2 transporter, were used to verify the action and inhibition of ABCG2. The LSC subpopulation was generally less than three percent of total cells, requiring analysis of large numbers of cells to generate statistically significant data. The extremely tight sample focusing provided by acoustic focusing cytometry enabled very high throughput while maintaining data integrity.

Poster Board Number: 1192

HYPOXIA INCREASES THE YIELD OF RETINAL CELL TYPES DIFFERENTIATING FROM MOUSE EMBRYONIC STEM CELLS AND IMPROVES THE MODELING OF RETINOGENESIS *IN VITRO*

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Retinal diseases affect more than 2 million individuals worldwide. Retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are the leading causes of permanent blindness and they are characterized by the progressive dysfunction and death of the light sensing photoreceptor cells of the retina. Due to the limited capacity of regeneration of the mammalian retina the scientific community has invested significant efforts in trying to obtain photoreceptors from embryonic stem cells (ESC). These represent an unlimited source of retinal cells, but it has not yet been possible to achieve specific populations, such as photoreceptors, efficiently enough to allow them to be used safely in the future as Cell Therapy of RP or AMD. The aim of our work is to study the possibility of obtaining a high yield of photoreceptors from directed differentiation by recapitulating development. We are therefore presenting a protocol of differentiation, involving hypoxia and taking into account extrinsic and intrinsic cues. These include niche-specific conditions as well as the manipulation of the signaling pathways involved in retinal development. Our protocol involves the directed differentiation of reporter cell lines generated from mESC, using a small molecule based-protocol in a chemically defined medium, as well as the control of the microenvironment, focusing particularly on controlling the oxygen tension.

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Our results show that hypoxia promotes and improves the differentiation of embryonic stem cells towards photoreceptors. Expression of Rhodopsin and Recoverin is increased under hypoxia condition, as shown by the techniques of immunofluorescence and qPCR. Furthermore, our protocol achieves the expression of various transcription factors associated with the different retinal phenotypes occurring during retinogenesis, in a temporal fashion, that corresponds with the known mammalian cues of retinal development. We have found expression of early eye-field development markers, as well as mature RPE and photoreceptor markers, such as Rx1, Otx2, Pax6, Chx10, MIFT, Crx, Nrl, Rhodopsin, Recoverin and RPE65 in these cells. We believe our results will support the hypothesis that hypoxia is necessary to induce the efficient differentiation of embryonic stem cells towards retinal phenotypes. Purification of these specific retinal progenitors will allow us to define conditions to expand a homogenous population that will be further differentiated into fully mature photoreceptors.

Poster Board Number: 1194

NEW INDUCTION METHOD OF PHOTORECEPTORS FROM MOUSE PLURIPOTENT STEM CELLS

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Retinitis pigmentosa is a major cause of visual loss. In the patient's retina of this disease, photoreceptors that convert light signals into electric signals degenerate initially because of his/her gene mutation. There is no clinical treatment for it now. Cell therapy is one possible solution for this disease. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are promising candidate sources for cell therapy. We previously succeeded in generating retinal cells including photoreceptors from mouse, monkey and human ESCs / iPSCs. However, with the previous method we could not obtain photoreceptors in high efficiency even after long time culture. In this study we improved the method for photoreceptor differentiation from pluripotent stem cells. First, we used Rx-GFP knocked-in mouse ESCs to obtained retinal progenitors and cultured them in various media containing protein or chemical factors involved in cellular signal pathways. We found that a chemical inhibitor of intracellular signal promoted retinal progenitor generation. Moreover we produced a mouse iPSC line from Nrl-GFP transgenic mouse to investigate photoreceptor differentiation. GFP-positive cells induced from the iPSC line expressed rod photoreceptor markers, rhodopsin and recoverin. By using this iPSC line, we confirmed that the induction method with the cell signaling inhibitor generated photoreceptors more efficiently than previous methods. This new induction method might bring photoreceptor transplantation closer to clinical therapy.

Poster Board Number: 1196

GENERATION OF STRATIFIED SQUAMOUS EPITHELIAL CELLS FROM MOUSE INDUCED PLURIPOTENT STEM CELLS

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In regenerative medicine of damaged corneal epithelium, in addition to the transplantation of donor cornea, a method to transplant the cultivated corneal epithelial sheet, which engineered using corneal epithelial stem cells isolated from limbal area of the cornea, has been established. Regenerative medicine using such donor-derived cells and tissues, somatic stem cells and ES cells include problems such as immunological rejection and ethical objection. Application of induced pluripotent stem (iPS) cells to regenerative medicine is expected to bypass these problems. To apply iPS cells to corneal epithelial disorders, we examined differentiation of mouse iPS cells into corneal epithelial cells. To promote epithelial differentiation, we use SDIA (Stromal cell-derived inducing activity) method. Mouse iPS cells were cultured on Mitomycin C-treated PA6 feeder cells with use of BMP-4 and serum stimulation. We found the derivation of epithelial colonies, which include the epithelial cells positive for an early ectodermal marker K18 and stratified epithelial markers K14 and p63. Clusters of the cells positive for corneal epithelial marker K12 were also found in K14-positive colonies. Based on the expression of K14, we found the derivation of epithelial cells was most effective when BMP was added during culture days 3-5. After several subcultures, we obtained K14-positive clones. Stratified epithelial sheets were engineered from an iPS-derived K14-positive epithelial clone by air-exposed culture with feeder cells in SHEM, on culture insert. In the resulting 3D sheet, along with the progression of differentiation by air-exposed culture, the expression of K18 was down-regulated and the expression of epidermal keratinocytes marker K10 was up-regulated instead. The expression of p63 at a high level was found in basal layer and that expression was down-regulated in suprabasal layers. The expression of K15, a stem cell marker for stratified epithelial cells, was found in basal layer as well. E-cadherin expression was found throughout the sheet. K12-positive cells disappeared during subcultures. To examine differentiation on mouse cornea further, cells labeled with Venus or mRFP were transplanted on denuded mouse cornea. The cell transplanted eyes were excised after engraftment and cultured *ex vivo*. Proliferation of the transplanted cells onto denuded mouse cornea was found *ex vivo* when cultured in serum containing-medium. Expression patterns of those differentiation markers in stratified epithelium formed on the denuded cornea were quite similar with that in the cultivated sheet on culture insert. Even though the expression of corneal epithelial marker K12 was not recovered, these results suggest that the culture method is available to produce epithelial cells from iPS cells, which can be engineered to produce polarized stratified cell sheets.

Poster Board Number: 1198

MODELING THE ZEBRAFISH CMZ, A LIFELONG RETINAL STEM CELL NICHE

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Zebrafish eyes are notable for their active retinal stem cell (RSC) niches, present throughout the life of the organism. These proliferative niches confer the zebrafish's remarkable ability to expand and repair the neural retina even in adulthood. The circumferential marginal zone (CMZ) is the primary source of new neurons added to the zebrafish retina. Interestingly, a CMZ-like region is present and may be active in the adult primate retina. While the zebrafish CMZ has been characterized developmentally, little is known about its function in the juvenile or adult animal. Furthermore, no model exists to describe the three-dimensional ontogeny of the CMZ or its contributions to the retina. In order to more fully elucidate the function and comparative traits of the vertebrate CMZ, we present a working model of CMZ ontogeny in zebrafish. We have mapped the proliferating cells originating from the CMZ from the initial formation of the CMZ in development to adulthood, demonstrating previously unreported complexity in the 3-dimensional ontogeny of this population. We have also measured the changing activity of the CMZ, including the frequency, number and position of cells contributed to the retina, and their lineage commitment. Combining these data with morphological measurements and measures of cell turnover, we have constructed a mathematical model describing the changing activity of the CMZ and its function in building the neural retina over time. This model may have significant implications for the understanding of the regulation and function of adult neurogenesis in the retina, and will assist us in determining the mechanisms by which the activity of the CMZ is maintained throughout life.

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Poster Board Number: 1200

THE ROLE OF DMBX1 GENES DURING RETINAL PROGENITOR CELL CYCLE EXIT AND DIFFERENTIATION

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Neurogenesis during vertebrate retinal development is a highly conserved process. In some vertebrates, such as teleost fish, new retinal neurons are generated throughout life under homeostatic conditions or as a regenerative response to retinal damage. There are two populations of retinal progenitor cells (RPCs) that exist in the mature fish retina: RPCs in the ciliary marginal zone at the far periphery of the retina and RPCs scattered among cells of the inner nuclear layer (INL). During zebrafish development, most RPCs exit the cell cycle and differentiate between 2 and 3 days post-fertilization (dpf). We previously found that the transcription factor called diencephalon/mesencephalon homeobox1 (dmbx1), is an important regulator of this process. The dmbx1 gene belongs to a family of paired-type homeobox genes, which includes crx, vsx/chx10 and otx genes, and is represented by two paralogs in the zebrafish genome, dmbx1a and dmbx1b. Loss of function of dmbx1 by morpholino-based gene knockdown causes widespread defects in retinal growth and differentiation. We find that although RPCs progress through all phases of the cell cycle, they do so at a rate that is ~5-fold slower than wildtype RPCs. Thus, at 3 dpf when the vast majority of RPCs have differentiated in the wildtype retina, the loss of dmbx1 prevents cells from exiting the cell cycle at the right time. Using cell transplantation, we show that this RPC defect in dmbx1 knockdown embryos is cell-autonomous. Furthermore, while RPCs with reduced levels of dmbx1 are capable of expressing the cell cycle inhibitor cdkn1c, they are incapable of down regulating cyclin D1, suggesting that the normal function of dmbx1 is to repress cyclin D1 expression. Consistent with this hypothesis, over-expression of dmbx1 prematurely represses cyclin D1 expression in the retina. Thus, these results demonstrated that dmbx1 is a key regulator for controlling the proper timing of RPC cell cycle exit. We are currently investigating which subtypes of neurons dmbx1 genes are expressing in the INL. Further insight into the role of dmbx1 in regulating the cell cycle and differentiation of RPCs will aid in our understanding of the mechanism that ensures the persistent, life-long neurogenesis in the zebrafish retina.

Poster Board Number: 1202

AUTOLOGOUS BONE MARROW DERIVED STEM CELLS TRANSPLANTATION FOR HEREDITARY RETINAL DYSTROPHY

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Purpose: To evaluate the short-term (10 months) safety of a single intravitreal injection of autologous bone marrow stem cells (ABMSC) in patients with Retinitis Pigmentosa (RP) or cone-rod dystrophy. Methods: A prospective, phase I, nonrandomized, open-label study was carried out. The study was approved by the local and National Institutional Review Board (ClinicalTrials.gov Identifier: NCT01068561).Five patients with RP were included, age-gender: 31-F; 35-M; 23-F; 33-M; and 35-M. Evaluations were performed at baseline, and 1, 7, 13, 18, 22, and 40 weeks after intravitreal injection of 10 x 106 autologous bone marrow stem cells (0.1 ml). Patients presented best-corrected ETDRS visual acuity (BCVA) of 20/200 or worse at baseline. In addition to comprehensive ophthalmological examination, patients yielded: standard full field electroretinography (ERG - ISCEV Standard); kinetic visual field (Goldman); fluorescein and indocyanine-green angiography; and optical coherence tomography (OCT). Results: No adverse side effect due to the injection was observed. A slightly improvement on BCVA (1 line) was measured in 4 patients 1 week after injection and kept during follow-up. Three patients showed undetectable ERG responses in all visits, while one patient showed residual responses for dark-adapted standard flash stimulus (a-wave amplitude around 35 µV), which was kept recordable during the entire follow-up. One patient showed a small response (a-wave amplitude around 20 µV) recordable only at weeks 7, 13, 22 and 40. Visual fields showed no reduction on visible area (Goldman Standard V5e) for any patient, at any visit. No other changes were observed on OCT or fluorescein and indocyanine green angiograms. Conclusions: Intravitreal injection of ABMSC in eyes with advanced tapetoretinal dystrophies was followed by absence of detectable structural or functional retinal worsening over a period of 10 months. Further studies are required to investigate the role, if any, of bone marrow stem cell therapy for the management of retinal dystrophies.

Poster Board Number: 1204

PLURIPOTENCY AND TRANSGENESIS OF BOVINE ES-LIKE CELLS

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Isolation and characterization of ES cells (ESCs) lines from domestic animals have great potential for studying the regenerating medicine and would represent a useful tool for developing therapeutic reagents for tissue repair and regeneration. For example, ESCs from domestic animals would be useful for improving genetic traits and developing the animals for disease resistance as well as production of pharmaceutical protein. Since mouse ESCs were first established nearly 30 years ago, only monkey, human and rat ESCs have been established to date. Although several efforts were made to establish ESCs in domestic animals, the pluripotent cells which differentiate to all three germ layers have not been generated so far. Another type of induced pluripotent stem cells (iPSCs) have been generated from mouse, rat, human, monkey and porcine somatic cells by forced expression of several combinations of transcription factors (Oct 4, c-Myc, Klf4 and Sox2). In view of the probable significant contribution of ESCs to manipulate the genome and generate the transgenic offspring to replace various tissues and organs in xenotransplantation, it seems to be appropriate to use ESC-technology to genetic modification of domestic animals. We established immortalized bovine ES-like cells with a normal karyotype that exhibited similar characteristics to those of murine ESCs which express Oct 4, STAT-3, SSEA-1 and alkaline phosphatase (AP). We also confirmed the pluripotent activity of these cells which were able to differentiate *in vitro* to neural progenitors and endothelial or hematopoietic lineages using a combination of FGF, EGF and PDGF or FGF, SCF and oncostatin M in serum-free MEMα medium, respectively. In addition, to avoid the immune surveillance by NK cells, we generated bovine ES-like cells that expressed HLA-B-EGFP, and found that they gave rise to ocular cells. Those differentiated cells were exhibited the markers of retinal stromal cells, pigment epithelial cells and crystalline lens. Furthermore, bovine ES-like cells transfected with EGFP were incorporated into both the inner cell mass and the trophectodermal cells to develop the blastocysts *in vitro*. Taken together, our findings provide possible evidence for the targeted genetic manipulation of the bovine genome to produce the cloned donor animals for regenerative therapy.

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ETHICS AND POLICY

Poster Board Number: 1206

TRANSLATIONAL RESEARCH IN STEM CELL BIOLOGY: HOW CAN BIOETHICISTS HELP?

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Failures to translate preclinical research with non-human animals into therapeutics that work in humans are legion. For every successful attempt, there are hundreds if not thousands of failures. While the failures are expected - humans are, after all, different from non-human animals in all sorts of relevant ways - researchers and research institutions, including funders, have been seeking ways to improve translational success rates and so reduce the absolute number and the relative proportion of failures. What is missing from the growing literature assessing epistemological and methodological dimensions of stem cell research with non-human animals is an evaluation of the ethical and other non-scientific values at stake in debates about how to maximize knowledge translation from preclinical to clinical contexts. This presentation summarizes two of the central contributions bioethicists can make to these increasingly important debates: (1) bioethicists can provide a moral assessment of the scope and nature of failures of translation from preclinical to clinical contexts; and (2) bioethicists can engage debates about the justification of research with non-human animals, debates that involve animal rights and animal welfare considerations as well as the required evidence base for proper risk-benefit assessment in the transition from preclinical to human subjects research in biomedicine. These two contributions are critical not only to the moral evaluation of translational research efforts, but also to any attempt to engage in upstream 'benchside consultation.'

Poster Board Number: 1208

ETHICAL AND SOCIAL ISSUES ON HUMAN PLURIPOTENT STEM CELL RESEARCH: EXTRACTS FROM DELIBERATIVE MEETINGS OF STAKEHOLDERS AT KYOTO UNIVERSITY

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With the progress of human pluripotent stem cell (hPSC) science, the ethical and regulatory environment encountered by laboratory-based scientists has become more complex. To better understand the current status, we convened two meetings at the Center for iPS cell Research and Application (CiRA) at Kyoto University, and collected the opinions of stakeholders concerning the ethical, legal and social issues surrounding hPSC research. At the first meeting in 2009, 18 scientists working at the CiRA expressed their opinions on the environment surrounding the study of patient-derived induced pluripotent stem cells (iPSC). They presented many topics that are important but have not yet been properly deliberated, including the handling and storing of patients' cells and data, disclosure of the data to the donor patients and the publication of research conducted using patient-derived iPSC. They also discussed the points that need prompt measures. We held a second meeting in 2010 to discuss a particularly controversial issue - deriving gametes from hPSC - which seemed timely because the corresponding Japanese governmental guidelines were revised in May, 2010, to allow conditional germ cell production from hPSC. More than 12 stakeholders working at Kyoto University (most of whom worked at the CiRA) in the fields of hPSC research, developmental biology of animal gametes, intellectual property management, research strategy and public relations attended this meeting. In this report, we describe the issues encountered by stakeholders at the CiRA in relation to the environment surrounding hPSC research and their suggestions for addressing some of these issues.

Poster Board Number: 1210

AN UNDERGRADUATE CURRICULUM IN HUMAN DEVELOPMENTAL AND REGENERATIVE BIOLOGY

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Human Developmental and Regenerative Biology (HDRB) is an integrated life science concentration (major) at Harvard College that educates students on how human beings develop from a fertilized egg, are maintained and repaired throughout adulthood, and age till life's end. Students are given a broad liberal education in modern life sciences by studying important biological principles of general relevance within the specific rubric of the developing and regenerating body. By adding an explicit and heavy emphasis on hands-on research opportunities in all four undergraduate years, HDRB has engaged and retained students with an interest in research while taking advantage of Harvard's unique strengths as a teaching college and research university.

Poster Board Number: 1212

ACCESS TO AND USE OF HESC LINES BY STEM CELL SCIENTISTS

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The potential of human embryonic stem cells (hESCs) to differentiate into many different cell types has inspired hope that these cells will lead to a new era of regenerative medicine. Yet at the same time, ethical controversy surrounding the derivation of these cells has inspired countless policy debates and led to a heterogeneous and uncertain policy environment. The unusual policy environment facing hESC scientists could plausibly affect the development of this field in many ways. This project focuses on one such potential impact - scientists' ability to acquire hESC lines for their research and their choice to use specific hESC lines. Drawing on data collected from approximately two hundred hESC scientists in the United States in a recent survey, this study identifies challenges scientists face when acquiring hESCs. In all, nearly 40% of hESC scientists surveyed reported that they faced an excessive delay acquiring one or more hESC lines and more than 25% reported that they were unable to acquire a hESC line that they wanted to study. Scientists' free-text descriptions of the difficulties they faced acquiring hESC lines suggest that prolonged negotiations over material transfer agreements and atypical levels of internal bureaucracy, driven by the contentious nature of the field, account for many of the problems scientists have acquiring hESCs. Analysis of the hESC lines that scientists use in their research indicated that a small number of different lines accounted for a large majority of all cell lines in use. In addition, scientists typically indicated they used only a small number of hESC lines in their research. More than 50% of hESC scientists reported that they used only one or two hESC lines and more than 75% of hESC scientists indicated that they used no more than three hESC lines in their labs. These results highlight the challenges hESC scientists face as they acquire cell lines for their research and suggest the development of hESC research may be hindered by an overreliance on a small number of cell lines and a shortage of labs equipped to conduct comparative hESC research.

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Poster Board Number: 1214

STEM CELL RESEARCH IN THE US AND UK: THE EFFECTS OF INTERNATIONAL COLLABORATIONS AND POLICY DISPARITIES ON RESEARCH

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Stem cell research is being conducted today in an increasingly global environment. Research articles resulting from multi-nation collaborations in all sciences have nearly doubled over the past ten year to 180,783 (accounting for over 20% of total publications). While it is generally assumed that international collaboration enhances the quality of research, this phenomenon has not been well examined. Stem cell research is unique in that it is a research area that faces policy disparities yet also often generates cross-border collaborations. Thus, studying international collaborations in stem cell research elucidates the role of existing international networks in promoting quality research, as well as the effects of disparate national policies on collaboration. This study examined the impact of collaboration on publication significance in the United States and the United Kingdom, two world leaders in stem cell research with differing policies. We reviewed publications by US and UK authors from 2008, along with their citation rates and the political factors that may contribute to the frequency of international collaborations. Our data, consistent with previous research on all sciences, found that UK researchers collaborate with international partners significantly more than US scientists. Fifty-three percent of total UK stem cell publications were the result of international collaborations versus 27% of total US stem cell publications. We also demonstrated that international collaborations significantly increase an article's impact, which was measured using citation rates. For US authors this was most apparent when the American was the corresponding author. The United States was the top collaborator for UK-authored articles, with Germany, Italy, the Netherlands, and France rounding out the top five. Four of the top collaborators with the United States – Germany, UK, Canada, and China – were consistent with previous studies on all science publications (including natural sciences, engineering and social sciences). Our results illustrate that collaborations across national borders had a positive effect on citation rates. The data also suggest that national stem cell policy differences and regulatory mechanisms driving international stem cell research in the US and UK did not affect the frequency of international collaborations, or even the countries with which the US and UK most often collaborated. Geographical and traditional collaborative relationships were the predominate considerations in establishing international collaborations.

Poster Board Number: 1216

PRODUCTION AND QUALITY SYSTEMS AT THE MASSACHUSETTS HUMAN STEM CELL BANK

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Advancement of the field of stem cell biology and regenerative medicine will depend on sustained efforts in a number of areas including but not limited to; (1) derivation and distribution systems, (2) streamlined materials transfer and licensing pathways, (3) regulatory agency influences and standardization of testing methods, and (4) development of effective information channels. The Massachusetts Human Stem Cell Bank seeks to develop and implement systems to influence these functional areas and facilitate growth in the field wherever possible. Funded by the Massachusetts Life Sciences Center, the Massachusetts Human Stem Cell Bank is located at University of Massachusetts Medical School and is a repository for human ES and iPS cell lines that are derived in laboratories worldwide. The Bank is equipped with a full spectrum of "state-of-the-art" equipment and employs comprehensive Standard Operating Procedures (SOPs) for cell culture, quality characterization, cryopreservation and distribution to both academic researchers and commercial operations throughout the world. We also provide training on hESC and iPS cell culture and cryopreservation techniques. The Bank currently distributes five hESC lines and two iPS lines and is in the process of acquiring more hESC and iPSC lines derived from either healthy or disease-specific host source material. To ensure the differentiation potential of the banked lines, the Bank currently performs *in vitro* lineage differentiation assays for some lines while testing the embryoid body (EB) formation potential for all the distributed cell lines. In addition, the Bank implements rigorous quality control systems to ensure that cells distributed are of the highest quality. To meet the needs of this rapidly evolving field, the bank is also engaged in testing and refining emerging techniques. For example, the Bank has successfully cultured both hES and iPS cells feeder-free for more than ten passages without changing the karyotype and pluripotent potential of these cells as measured by EB formation and analysis of gene expression profiles by Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-QPCR) & ICC assays. In addition, we have completed shipping studies to ensure optimal recovery upon receipt. Our cell line Catalog, cell culture and characterization SOPs, cell deposit & request documentation can all be found at our Website: http://www.umassmed.edu/MHSCB/index.aspx.

Poster Board Number: 1218

EVOLUTION OF STEM CELL RESEARCH 2004-2009: A CITATION ANALYSIS PERSPECTIVE

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This study examines the development of the international stem cell (SC) research field from 2004 to 2009 by analyzing the evolution of its intellectual structure and key researchers during this time. Methodology: We perform author citation and cocitation analyses for three 2-year slices of this 6-year period based on a nearly complete and highly cleaned dataset of the stem cell literature (including cited references) compiled from the PubMed and Scopus databases. For each time slice, a set of key researchers was identified as the 200 most highly cited during that time period, using automatic author name disambiguation to work around the extreme author name ambiguity problems in this field - a first in the field of scientometrics. Based on their co-citation patterns with other researchers, these researchers are grouped into specialties using the Factor Analysis statistical procedure, and the interrelationships between researchers and specialties are visualized using the Pajek network analysis program. The research specialties that these authors represent are manually inferred from the topics of their highly cited publications. Preliminary Results: The main separation into subfields of the stem cell research field appears to be fairly stable over the six years, but some major development trends and revolutions during this time are identifiable, as are some interesting details. The main subfields we identified throughout are (1) embryonic and pluripotent SCs (biology, molecular biology, and biotechnology); (2) neural stem cells, especially adult neurogenesis; (3) medical stem cell research and applications, ranging from cancer to regenerative medicine; and (4) a number of foundational cell biology areas that stem cell research appears to draw upon. Major revolutions that we can identify from this analysis include (a) the apparently complete failure of an initially strong attempt to unify the field under the banner of stem cell plasticity; (b) the emergence of the cancer stem cell concept; and (c) the recent induced pluripotent stem cell breakthrough. More gradual trends within the field are not as clearly identifiable, but there are a few observations that merit mention. (a) While early attempts to rally the field around the concept of SC plasticity apparently failed, there does appear to be a gradual trend both towards improved cohesion in research tools and methods and towards a deepening understanding of a common nature of stem cells. (b) While haematopoietic

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SCs continue to play a major role in both research and medical practice, the interest in their study for their own sake appears to be fading. Among the interesting details that this analysis suggests, we mention (a) the merging of the myocardial repair specialty into the vascular growth area rather than with the muscle stem cell field, indicating perhaps a greater promise in considering the heart as a rather large blood vessel than as a muscle when treating heart attacks; (b) the evolution of a mesenchymal SC specialty towards a focus on cytotherapy; and (c) the recent appearance of a small and vague specialty concerned with the biotechnology surrounding knockout mice at around the same time that the Nobel Prize went to the discoverers of this important biomedical research tool. Conclusions: In all, we observe a rapid evolution of stem cell research within a relatively stable framework. There are signs that the field is maturing and evolving towards a cohesive view of its subject matter.

Poster Board Number: 1220

SURVEY OF 1,200 HUMAN ES AND IPS CELL LINES

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Since the first derivation of human embryonic cell (hESC) lines in 1998, progress in this field has been rapid and has resulted in the derivation of more than 1,200 cell lines. Furthermore, the advent of induced pluripotent stem cell (iPSC) technology has transformed the stem cell landscape. To assess the current state of the pluripotent stem cell field, we have surveyed 1,200 hESC and induced pluripotent stem cell (iPSC) lines using information compiled at the International Stem Cell Registry (http://www.umassmed.edu/iscr). Validated unpublished information, as well as data from over 1,600 published studies was analyzed. Parameters in this study include: genetic disorders, genetic manipulation, genomic stability, gender, feeder and feeder-free culture conditions, and differentiation protocols. The results from this study provide an informative timeline for overcoming obstacles to regenerative medicine, and suggest intriguing questions for future experiments.

MUSCLE CELLS

Poster Board Number: 1222

TRANSDIFFERENTIATION BY BACTERIAL MEDIATED MYOD PROTEIN DELIVERY

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Forced exogenous gene expression has been well characterized as an effective method for directing both cellular differentiation and dedifferentiation. However, transgene expression is not amenable for therapeutic application due to the potential for insertional mutagenesis. Protein based techniques provide a safe alternative, but current protein delivery methods are quite limited by labor-intensive purification processes, low protein yield and inefficient intracellular targeting. Such limitations may be overcome by using a naturally occurring bacterial protein injection system. Pseudomonas aeruginosa utilizes a Type III Secretion System to inject bacterial proteins directly into the eukaryotic cell cytoplasm. Our previous studies describe the ability of this system to easily deliver a high quantity of protein to both differentiated and pluripotent cells using a genetically attenuated strain. Utilizing Cre recombinase as a reporter, we demonstrate high frequency loxP mediated recombination in the chromosome of the recipient cells, suggesting the protein is not only efficiently targeted to the nucleus, but also retains its biological function. MyoD is a key muscle regulatory factor, the overexpression of which is able to induce transdifferentiation of numerous cell types, such as fibroblasts, into functional myocytes. Here we demonstrate transient injection of MyoD protein by P. aeruginosa is sufficient to induce myogenic conversion of mouse embryonic fibroblasts. In addition to clear morphological changes, muscle specific gene expression has been observed by immunostaining as well as RT-PCR. These studies serve as a foundation for the bacterial delivery of transcription factors to efficiently modulate concentration-dependent and temporal activation of gene expression to direct cell fate without jeopardizing genomic integrity.

Poster Board Number: 1224

MOUSE MODEL TO UNDERSTAND THE ROLE OF DUX4 IN FSHD

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FSHD is an autosomal dominant disease that affects 1:20000 individuals. Mapping studies have associated the disease with a reduced number (1-10) of the D4Z4 macrosatellite repeats from the usual ~100. These repeats lie adjacent to the telomeres and are usually present in a highly silenced epigenetic state. It is not clear which genes are affected or how DNA methylation patterns affect the disease. Within the D4Z4 repeat is an ORF encoding a putative transcription factor named Dux4, containing two homeodomains. Although the function of Dux4 is unknown, the Dux4 homeodomains are similar to those of Pax7, a protein known to be involved in muscle development, proliferation and differentiation. We have previously reported that Dux4 is toxic when misexpressed at high levels in many cell types, and blocks differentiation of myoblasts when expressed at low levels, and competes with Pax7 for regulation of myogenic target genes. To model Dux4 function *in vivo*, we made a Dux4-inducible mES cell line by inserting a doxycycline-inducible Dux4 allele (iDux4+3'UTR) onto the X chromosome at a euchromatic region (HPRT). High-level induction of the Dux4 was toxic to mES cells but low-level Dux4 resulted in altered differentiation. When iDux4+3'UTR mice where generated and bred, this allele demonstrated leaky phenotypes in females, and male-specific lethality. Rare live-born males were small and underdeveloped with abnormal skin and defective sperm development and showed changes to muscle fibers, but no overt muscle degeneration. However, mice died within 1 month, well before the stage degeneration usually begins in FSHD. Dux4 protein could be induced and observed in cultured primary cells, and we are evaluating pups and embryos for Dux4 expression *in vivo*. Dux4 carrier females were smaller and displayed the skin phenotype in transverse stripes. We hypothesize that the 3' UTR contains an enhancer which drives leaky expression in some embryonic cell types and that X chromosome inactivation combined with selective survival of XDUX4-inactive cells protects the females from the lethality. To test for selectively biased X inactivation, we crossed Dux4 carrier females with XGFP males. Upon FACS analysis of the GFP+ cells in XGFP/X+ vs. XGFP/ XDux4 female progeny, we found that the latter had an elevated frequency of GFP positive cells in most tissues, including the satellite cell compartment of skeletal muscle, confirming our hypothesis of selective XDUX4 inactivation. This mouse model suggests that Dux4 is a dominant lethal gene even when expressed at very low levels and can cause a variety of developmental defects in EBs and in embryo development.

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Poster Board Number: 1226

MYOSTATIN DEPENDENT MUSCLE REGENERATION IN EXPERIMENTALLY INDUCED DIABETES

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Insulin insufficiency following the depletion of insulin producing pancreatic β-cells or peripheral insulin resistance cause diabetes mellitus, a severe metabolic disorder that affects many organ systems and results in poor wound healing. In this work, we studied the direct influence of acute insulin insufficiency and deregulated glucose metabolism on the regenerative potential of skeletal muscle stem cells (satellite cells). Our results establish that in an animal model of type 1 diabetes, muscle stem cells fail to activate within 72 hours of high glucose blood levels, and repair of young muscle instantly deteriorates, resembling that of old tissue. Furthermore, our data demonstrate that this deleterious effect of insulin insufficiency on organ stem cells is direct and caused by a significantly increased TGF beta receptor signaling in satellite cells. Importantly, this work establishes that the increase of TGF beta receptor signaling and poor regenerative capacity can be restored to healthy levels in muscle stem cells *in vivo* by the administration of follistatin or an Alk5 inhibitor. Notably, transient Alk5 inhibitor treatment does not reduce the levels of blood glucose in insulin depleted mice, i.e. does not cure diabetes itself, but rather restores satellite cell responses through a TGF-beta signaled cell-cycle halt by CDK inhibitors. Summarily, the regenerative responses of muscle stem cells rapidly fail without insulin due to increased TGF beta receptor signaling; even a brief lack of insulin and perturbed glucose metabolism have immediate negative consequences for tissue maintenance and repair, and follistatin or Alk5 inhibition is predicted to rescue tissue regeneration in the presence of diabetes.

Poster Board Number: 1228

ABERRANT GENE EXPRESSION IN FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY INDUCED PLURIPOTENT STEM CELLS

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Facioscapulohumeral (FSH) muscular dystrophy is a common autosomal dominant neuromuscular disease for which there is no effective treatment. FSH is associated with heterochromatin loss and a gain-of-function mutation on human chromosome 4q. The mutation creates a polyadenylation site that stabilizes transcripts of DUX4, a double homeobox gene of unknown function. Previous studies suggested that DUX4 may be toxic and/or a transcriptional activator. Our goal is to determine the relationship between DUX4 expression and the pathology observed in FSH. In this study, we established an *in vitro* model of FSH using human induced pluripotent stem (iPS) cells. We used the model to determine: 1. the expression of DUX4 in FSH and control embryonic and adult cells and 2. the changes in gene expression associated with DUX4 expression. iPS cells were isolated from control and FSH skin and muscle biopsy tissue using retroviral vectors to express OCT4, SOX2, KLF4, and c-MYC. Both fibroblast- and myoblast-derived iPS cells express typical stem cell markers, have normal karyotypes, and form teratomas containing differentiated cells from all three embryonic germ layers. To determine whether DUX4 is expressed in FSH-iPS cells, we performed RT-PCR and SYBR green quantitative PCR using probes specific to the DUX4 transcript produced from the mutant allele. DUX4 transcripts were detected in all FSH-iPS cell lines but absent in most control iPS cell lines and human embryonic stem cells. Expression of DUX4 in pluripotent cells was correlated with 4q haplotypes containing the polyadenylation signal. To determine whether DUX4 alters gene expression in FSH-iPS cells, we performed microarray analysis (Affymetrix). Gene expression was largely similar in FSH- and control-iPS cells prior to differentiation with several notable exceptions. Our results suggest that DUX4 is aberrantly expressed in FSH pluripotent cells of the embryo and adult somatic cells and may reflect a pathogenic role in early development or renewable stem cell populations such as satellite cells in mature muscle.

Poster Board Number: 1230

PROLIFERATION AND CLONAL CHARACTERIZATION OF HUMAN MUSCLE DERIVED CD133 POSITIVE STEM CELL DEFINE AN INTRINSIC HETEROGENEITY

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In the muscle there exist different stem cell populations that are able to participate in muscle regeneration. How muscle stem cells balance their self-renewal capacity and their ability to differentiate are central questions in stem cell research. Moreover, recent findings supported the notion that heterogeneity is a hallmark of stem cells. It is known that these cell populations express a variety of surface markers including CD29, CD56, CD44, CD90, CD105, STRO1. Here we have focused our attention on the so-called MDSCs (Muscle Derived Stem Cells) that can be rather considered as satellite precursors. In the past our group focused its attention on a stem cell population expressing CD133 antigens on its surface. The analysis of muscle-derived CD133 positive cells showed that they have been characterized for their regenerative potential *in vivo*, as well as their ability to repopulate the satellite cell niche. However, we found in the CD133 positive MDSCs a phenotypic heterogeneity in the presence of some surface antigens: in the same cell population we found an oscillation of expression of endothelial, myogenic and mesenchymal markers. Based on these assumptions we sorted some cell subpopulations obtained from muscles of healthy orthopedic patients: mesenchimal like cells (133pos 73pos 44pos 29pos 34neg 45neg); endothelial like cells (133pos 90pos 146pos 31pos 45neg) and myogenic like cells (133pos 56pos 45neg 34pos or neg). These subpopulation of sorted CD133 were cloned and analyzed for their capacity to proliferate and differentiate into the endothelial and myogenic lineages. In these experiments we found stem cell properties in the mesenchymal like CD133 positive clones. In fact these cells possess clonogenic potential and well differentiate into endothelial and myogenic cells. In conclusion, our study showed that muscle derived CD133 positive cells represent a heterogeneous population of mesenchymal, myogenic and endothelial progenitors. Further experiments are needed in order to understand whether CD133 purification could be useful for clinical applications in muscular dystrophies.

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Poster Board Number: 1232

THE LIN28/LET-7 AXIS REGULATES EMBRYONIC AND ADULT GLUCOSE METABOLISM IN MAMMALS

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The let-7 family of tumor suppressor microRNAs repress oncogene translation and promote differentiation in embryonic stem cells. The RNA-binding proteins Lin28A and Lin28B block the processing of let-7 family members to promote pluripotency and malignancy. Here we demonstrate an unexpected role for Lin28a/b and let-7 in regulating embryonic and adult glucose metabolism in mammals. Loss of Lin28a in E10.5 mouse embryos and inducible overexpression of let-7 in mouse embryonic stem cells reduce both glycolytic flux and NADH levels, as shown by metabolomics. In contrast, overexpression of Lin28a in adult myoblast cells increases glycolytic flux and NADH levels. When overexpressed in transgenic mice, both Lin28a and LIN28B promote a metabolic state with high glucose uptake and high insulin sensitivity that resists obesity-induced diabetes, by activating the PI3K-mTOR pathway. Overexpression of let-7, on the other hand, leads to impaired glucose uptake, due to let-7-mediated repression of the PI3K-mTOR pathway. *In vitro* and *in vivo*, the enhanced glucose uptake and insulin-sensitive phenotype conferred by Lin28a is abrogated by the mTORC1 inhibitor rapamycin. In addition to interactions with the PI3K-mTOR pathway, let-7 targets numerous genes associated with fasting glucose and type-2 diabetes, based on human genome-wide association studies. Furthermore, mRNA transcripts associated with the Lin28a RNA-binding protein in embryonic stem cells are also significantly enriched for glycolysis enzymes. These data establish the Lin28/let-7 pathway as a central regulator of both embryonic and adult mammalian glucose metabolism, with implications for the theory of fetal origins of metabolic disease (Barker's hypothesis), and the Warburg effect in cancer.

Poster Board Number: 1234

CHARACTERIZATION OF ROLE OF BAF60C IN TGF-β MEDIATED SMOOTH MUSCLE INDUCTION IN RODENT BONE MARROW DERIVED ADULT STEM CELLS

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Smooth muscle cells (SMC) occur in two varieties: vascular SMC in blood vessels and visceral SMC lining the visceral organs. The main function is to regulate blood pressure (vascular) and movement (visceral). Developmentally, SMC can be derived from multiple sources: neural crest, local mesenchymal tissue, splanchnic mesoderm, as well as derived by transdifferentiation of endothelial cells in blood vessels. Development of SMC is critical for the viability of embryo. In the adult, there are many diseases associated with SMC dysfunction, such as asthma atherosclerosis, obstructive bladder disease, gastrointestinal and reproductive disorders. SMC are a unique type of cells that have the ability to phenotypically remodel during injury and disease. However, there is very little known regarding the genes, specifically transcription factors (TFs), and signal pathways necessary for SMC development and / or that regulate remodeling of SMC during injury and in disease conditions. Moreover the lack of proper model systems to study SMC development *in vivo* (as underdeveloped SMC leads to embryo lethality) has impaired our advancement in understanding SMC development. The aim of this study is to use adult bone marrow derived stem cells as model to identify genes involved in smooth muscle generation. We differentiated rat Multipotent adult progenitor cells (MAPC) towards smooth muscle using TGF-β and PDGF-BB, following which we preformed a temporal genome-wide transcriptional analysis to identify novel regulators of smooth muscle genes. We identified, among others, Baf60c as a transcription factor that regulates certain smooth muscle genes. Baf60c or Smarcd3 is a SWI/SNF chromatin remodeling complex protein shown to be important in cardiac and skeletal muscle genesis. Lickert et al. in 2004 had demonstrated that silencing of Smarcd3 expression leads to defects in developing heart. Ochi et al. had shown that morpholino mediated knock down of Smarcd3 leads to delay in onset of skeletal myogenesis. We here demonstrate that Smarcd3 is a target of Smad mediated TGF-β signaling. In addition, inhibition of Smarcd3 leads to a decrease in TGF-β mediated smooth muscle gene induction. Recently, Takeuchi et al. (2009) demonstrated that Baf60c in association with cardiac specific factors could differentiate mouse mesoderm to heart tissue. Similarly, we observed that Smarcd3 was sufficient to induce SMC genes including smooth muscle actin (SMA) in rat MAPC in serum and cytokine free conditions, albeit with lower efficiency. We are currently characterizing the associated proteins that aid Smarcd3 in inducing a SMC fate in rat MAPC.

Poster Board Number: 1236

SCREENING FOR CO-ACTIVATORS OF MYOCARDIN: NEW DISCOVERY IN MECHANISM OF REGULATION OF SMOOTH MUSCLE CELL DIFFERENTIATION

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The proliferation and differentiation of smooth muscle cells (SMCs) play critical roles in vascular diseases. Since myocardin is a key factor in smooth muscle differentiation, in this experiment we were looking for transcription factors (TFs) or co-activators in smooth muscle differentiation signaling pathway by screening the proteins that could bind and interact with myocardin. By studying the function of the protein candidates, we may discover new molecular mechanisms of smooth muscle differentiation. After screening of 1,070 transcription factors by luciferase assay and real-time PCR, we discovered that a list of 11 TF candidates, such as zbtb43, rxra, rai14, maged1, foxo3a, could increase luciferase activity by interacting with myocardin and also could be highly expressed in mouse aorta and human SMCs in a mRNA level. We successfully constructed the Myc-Tag expression plasmids of some of the TF candidates and further confirmed by co-immunoprecipitated assay (co-IP) that myocardin could bind with 4 TFs (RAI14, MAGED1, ZBTB43 and FOXO3A). Moreover, the expression level of above 4 TFs was significantly upregulated during the process of mouse embryonic stem cells differentiating into SMCs induced by 10-5M all trans-retinoic acid. All these data suggested that these newly discovered TFs might play an important role in the SMC differentiation signaling pathway though interacting with myocardin.

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Poster Board Number: 1238

ADULT SKELETAL MYOGENESIS IN A RAT MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Objective: Considering the future development of neuromuscular reconstruction therapy in amyotrophic lateral sclerosis (ALS), we investigated a temporal profile of endogenous myogenesis in skeletal muscles of a rat model of ALS. Background: ALS is a progressive and fatal neurodegenerative disease characterized by adult-onset loss of motoneurons without effective treatment to date. Axonal retraction from innervating skeletal muscles has been reported as an early pathology in ALS patients and in transgenic (Tg) mice with ALS-linked mutant SOD1 genes. Methods: We examined endogenous myogenesis in hindlimb skeletal muscles of His46Arg mutant SOD1 Tg rats at pre-symptomatic, early symptomatic, and late symptomatic stages (n=4-5) with their age-matched non-transgenic littermates (non-Tg). Continuous administration of bromodeoxyuridine for 7 days labelled newborn cells *in vivo*. After the administration, we performed multiple immunofluorescence using various myogenic markers including MyoD, neonatal myosin heavy chain, and neural cell adhesion molecules in cryosections of femoral quadriceps and anterior tibial muscles. For quantification, we captured digital images of the immunofluorescence under confocal laser-scanning microscopy. Results: In contrast to non-Tg, the Tg rats showed a significant increase of myogenesis with a distinct expression of the myogenic proteins, progressive neurogenic myopathology, and axonal retraction from neuromuscular junction. The myogenic response in Tg rats peaked at the early stage of disease, followed by decrease at the later stage. Furthermore, chondroitin sulfate was accumulated progressively in the interstitial space in Tg rats. Conclusions: The present results revealed an intrinsic regenerative response against the chronic denervative amyotrophy in the ALS-like disease. The response, however, does not persist sufficiently throughout the disease. In addition to motoneuron restoration, promoting myogenesis and reinnervation to skeletal muscles could be needed for inhibiting amyotrophy and neuromuscular reconstruction.

Poster Board Number: 1240

BIODEGRADABLE L LACTIDE E CAPROLACTONE COPOLYMER SCAFFOLD SUPPORTS MYOBLAST GROWTH AND DIFFERENTIATION

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Skeletal muscle regeneration involves the activation of satellite cells to myoblasts, followed by their proliferation and fusion to form multinucleated myotubes and myofibers. The potential of *in vitro* proliferated myoblasts to treat various diseases and tissue defects can be exploited using tissue engineering principles. With an aim to develop a biocompatible and biodegradable scaffold that supports myoblasts growth and differentiation, we have developed a scaffold with 85/15 L-lactide/ε-caprolactone copolymer (PLC) using a phase inversion combined with particulate leaching method. Biodegradability studies indicated that the highly porous scaffold retained its integrity for 5 months *in vitro* and had undergone complete degradation within nine months *in vivo*. The scaffold supported human myoblasts attachment and its proliferation. The myoblasts seeded on the PLC scaffold differentiated and fused to form multinucleated myotubes *in vitro*. Histological and molecular analyses of the PLC scaffolds loaded with GFP labeled myoblasts and implanted ectopically under the skin in SCID mice indicated the presence of multinucleated myotubes. Our results suggest PLC scaffolds loaded with myoblasts can be used for skeletal muscle engineering or muscle repair.

NEURAL CELLS

Poster Board Number: 1242

DIFFERENCES BETWEEN HUMAN EMBRYONIC STEM CELL (HESC) LINES IN CAPACITY FOR DIFFERENTIATION TO MESENCEPHALIC DOPAMINERGIC (MDA) NEURONS

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It is possible to induce hESC to undergo mDA neuronal differentiation by simple exposure to feeder cell lines, particularly the mouse PA6 stromal cell line; this effect is known as SDIA. We have previously identified four factors highly expressed by PA6 cells which are able to mimic SDIA. These were stromal cell-derived factor 1 (SDF-1/CXCL12), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2), and ephrin B1 (EFNB1), termed SPIE. All four factors are required for efficient mDA differentiation. SPIE-induced mDA neurons generated action potentials and formed functional synaptic connections. In the present study, we examined differences between hESC lines in their responses to SPIE, and compared these to mDA differentiation of the same hESC lines using the differentiation method reported by Yan et al. Five hESC lines were used, BG01, BG02, BG03, ES02, and ES04, as well as BG01V2 which differs from BG01 in that it has a trisomy at chromosome 17. BG01V2 and BG03 rapidly formed large numbers of mDA neurons in response to SPIE, while BG02, ES02, and ES04 did not respond effectively. In BG02, SPIE produced small numbers of neurons positive for tyrosine hydroxylase, but lacking the mesencephalic marker MSX1. SPIE also did not induce mDA differentiation of various mouse neural progenitor cell lines or Ntera2 cells. The Yan et al. technique for mDA neuron differentiation from hESC involves an initial embryonic body formation phase, followed by isolation of neural rosettes and treatment with SHH and FGF8. In contrast to the SPIE technique, the Yan et al. technique is effective for all hESC lines. BG01V2 and BG03 differentiated into mDA neurons in a period of 16 days, although BG03 differentiated less effectively than BG01V2. All other hESC lines required 45 days for mDA differentiation. We examined the possibility that differences in WNT signaling were responsible for the differences between hESC lines in mDA neuron differentiation. Using Western blotting, canonical and noncanonical signaling inhibitors (Dkk-1 and SP600125 respectively), and a chromatin immunoprecipitation (ChIP) assay, we examined the role of WNT at various differentiation stages. Noncanonical WNT signaling promoted the neuroectodermal fate through inhibition of hESC self-renewal while canonical WNT stimulated both neuroepithelial cell proliferation and mDA differentiation. Inhibition of WNT signaling reversed the accelerated differentiation of both BG01V2 and BG03. Because LMX1a is regulated by the β-catenin pathway and modulates expression of transcription factors required for mDA differentiation, we performed a ChIP assay. Binding of β-catenin to the promoter of LMX1a was greater for the rapidly-differentiating lines BGO3 and BGO1V2, as compared to the other lines. Therefore, differences in WNT signaling can account for the differences between hESC in their propensity to undergo rapid mDA differentiation. These data also confirm the role of canonical WNT signaling in mDA differentiation.

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INHIBITION OF NOTCH SIGNALING PREVENTS NEURAL OVERGROWTH AFTER THE TRANSPLANTATION OF NEURAL PROGENITORS DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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When transplanted, pluripotent stem cell-derived neurons survived and contributed to functional recovery in animal models of Parkinson's disease; however, their transplantation has always been accompanied by a risk of tumor formation. Removal of undifferentiated cells from the donor population with fluorescence-activated cell sorting (FACS) was successful in preventing tumorigenesis; however, immature neural progenitors in the graft often continued dividing instead of terminally differentiating into mature dopaminergic neurons. Such unpredictable growth of the graft, referred to as neural overgrowth, could pose a safety risk over time. Notch signaling pathway plays a role in maintaining the multipotency and proliferative capacity of neural progenitors. Gamma secretase is a protease complex responsible for notch signal transduction by cleaving notch intracellular domain upon binding of the ligands to the notch receptor. In this study, we investigated if a transient notch inhibition in human induced pluripotent stem cell (iPSC)-derived neural progenitors with gamma secretase inhibitors (GSIs) could control the growth of the grafts after transplantation. We first evaluated if GSIs could suppress the growth of the graft consisting of highly proliferative immature progenitors. Dopaminergic progenitor cells were generated after the 22 days of differentiation via the serum-free culture of embryoid body-like aggregates quick (SFEBq) method. Quantitative PCR, immunocytochemical and cell cycle analyses were used to assess the proliferation and maturation of those progenitors *in vitro*. A 4-day treatment with GSIs significantly reduced dividing population, and promoted the transition from PAX6-positive neural progenitors into MAP2AB-positive neurons. Next, they were transplanted into the striatum of NOD-SCID mice. Positive Nestin, NCAM and Tubβ3 stainings in the grafts indicated the neural and almost neuronal identities of the transplanted cells in animals of all groups. At 8 weeks post-transplantation, we observed prominent neural overgrowth with numerous neural rosettes in all animals of the control group (n=7). On the other hand, grafts pre-treated with GSIs were significantly smaller in volume compared to control. Such difference most likely to have reflected the reduced proliferative capacity of the transplants pre-treated with GSIs, as confirmed by a significant reduction in PAX6-positive and Ki67-positive populations at the time of sacrifice. Furthermore, stronger Tubβ3 staining indicated the promotion of neuronal maturation by GSIs. These results suggest that a transient notch inhibition with GSIs could be combined with other strategies such as FACS to optimize the safety of cell replacement therapies with iPSCs.

Poster Board Number: 1246

DIFFERENTIATION OF ADULT HUMAN SKIN-DERIVED PRECURSOR CELLS INTO DOPAMINERGIC NEURONS-LIKE PROGENY

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INTRODUCTION: Skin-derived precursor cells (SKPs) have been thoroughly studied since their discovery by Toma et al. a decade ago. These multipotent stem cells offer a great alternative to other more controversial sources of stem cells and their capacity to differentiate into mature neurons, highlights their therapeutic potential for a variety of neurological diseases, such as Parkinson's disease (PD). One aspect of PD is the extensive loss of dopamine (DA) neurons in the substancia nigra pars compacta, inducing a lack of DA secretion in the striatum. OBJECTIVE: The purpose of this study was to determine whether adult human SKPs could fully develop into functional dopaminergic-like neurons, a subset of cells particularly targeted in PD. METHODS: Skin samples used in these experiments were obtained from breast reductive surgery, and fully informed consent was obtained for each patient. Cells were extracted from skin biopsies using enzymatic digestion and cultured as floating spheres in a proliferation medium containing epidermal growth factor, leukemia inhibitory factor and basic fibroblast growth factor2. After a month of purification/proliferation in suspension, spheres were triturated and seeded on poly-d-lysine/laminin coated culture flask and cover slips. Differentiation process was divided in three stages depending on the cocktail of morphogens and neurotrophins used. Differentiated cells were analyzed regarding their generic and dopaminergic specific neuronal markers using immunofluorescence and flow cytometry techniques. PCR was used to analyze the expression of Nurr-1, a dopaminergic phenotype triggering gene. ELISA assessment of the secretion by the differentiated cells of brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and DA was also performed. RESULTS: Immunofluorescence and flow cytometry analyses showed that dopaminergic-induced cells express tyrosine hydroxylase (TH), a key enzyme in DA production (33% after 21 days of differentiation - D21). Cell imaging results also showed the expression of VMAT2 and DAT, two receptors respectively responsible for vesicular transport of DA to the synapse and re-uptake of DA. We also demonstrated that differentiated adult human SKPs secrete BDNF (~440 pg/ml at D21) as well as GDNF (~1800 pg/ml at D21), two important neurotrophins promoting neuronal cell survival. Finally, DA release (~12 ng/ml at D21) and Nurr-1 expression was also observed. CONCLUSION: The present study demonstrates that adult human SKPs can differentiate into dopaminergic-like neurons expressing several specific markers of this system. In addition, these cells secrete similar amounts of BDNF, GDNF and DA compared to dopamineric neurons. These cells could prove to be an autologous cell therapy option for the treatment of Parkinson's disease.

Poster Board Number: 1248

PARKINSON'S DISEASE PATIENT SPECIFIC IPS CELL-DERIVED NEURAL CELLS DEMONSTRATE GENOTYPE-SPECIFIC VULNERABILITIES *IN VITRO*

Isacson, Ole, Dawson, T. M., Feng, J., Krainc, D., Lee, V. M., Marder, K., Przedborski, S., Ross, O. A., Surmeier, D. J., Trojanowski, J. Q., Wszolek, Z. K., Cooper, O., Seo, H., Hargus, G., Deleidi, M., McLean, J., Sundberg, M., Kartunen, A., Brown, T., Perez-Torres, E., Clark, L., Moskowitz, C., Andrabi, S., Xie, Z., Guardia Laguarta, C., Mazzulli, J., Chen, L., Volpicelli-Daley, L., Romero, N., Jiang, H., Carrillo-Reid, L

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The greatest risk factor for Parkinson's disease (PD) is age but genetic and epigenetic sources of cellular stress can accelerate the age of disease onset. While kindreds and genome sequencing technology can estimate an individual's risk for developing PD, human cell models are required to understand the complex biological consequences of an authentic genetic risk load and develop treatments. As a new genetic tool to study PD, we generated iPS cell lines from 6 patients with SNCA, LRRK2 or PINK1 mutations and 2 healthy individuals. Next, we developed a comparative multiple hit model of PD by administering a dose range of PD associated toxins to iPS cell-derived neural cells. Cytotoxicity assays showed that LRRK2 R1441C patient neural cells are more vulnerable to valinomycin and MPP+ than neural cells derived

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from healthy individual iPS cells. Meanwhile, SNCA triplication patient neural cells were solely vulnerable to MPP+ and PINK1 p.Q456X patient neural cells to valinomycin alone. Furthermore, flow cytometry and immunocytochemistry indicated that a subtoxic dose of valinomycin but not MPP+, 6-OHDA or hydrogen peroxide increased the levels of mitochondrial superoxide and cell death of PINK1 p.Q456X patient neural cells. Interestingly, the concentration of valinomycin was 10-fold less than previously described to induce cellular stress in the same patients' fibroblasts. These data suggest that cellular reprogramming technology can provide a biologically-relevant cellular context for studying the genetic risks for PD.

Poster Board Number: 1250

SYNERGISTIC EFFECT OF PITX3 OVEREXPRESSION AND GDNF INDUCTION IN DOPAMINERGIC PROGRAMMING OF NT2, A HUMAN PLURIPOTENT EMBRYONIC CARCINOMA CELL LINE

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Cell replacement strategies are sought to replenish dying neurons by fresh and functional ones obtained from differentiation of potential sources ranging from stem cells to dopaminergic precursors. Human Nteratocarcinoma cell line NT2 expresses dopamine signals and, owing to its safety profile, has been applied for clinical transplantation. We have established a Pitx3-expressing NT2 cell line mediated by lentiviral transduction that stably overexpresses the dopaminergic transcription factor, as tested via RT-PCR analysis and monitored by GFP reporter expression. The engineered NT2 cells were cultured in a defined medium supplemented with 5% SR serum that induces embroid bodies (EB) formation. These structures gained sharp and defined borders following retinoic acid treatment. Next, we cultured these EB-differentiating cells in the presence of conditioned medium taken from various cell lines over-secreting neurotrophic factor GDNF. This step accelerated neuronal morphology of the cells. We also found that the EBs formed from our NT2 clones can differentiate better when trypsinized to grow as single cells. Currently, we are determining the neuronal fate of the differentiated cells using specific markers that assist identifying dopaminergic neurons. Our findings indicate that GDNF and Pitx3 act in synergy to induce neuronal differentiation.

Poster Board Number: 1252

PHENOTYPIC SCREENING USING HUMAN EMBRYONIC STEM CELL DERIVED NEURAL STEM CELLS AS A TRANSLATIONAL *IN VITRO* MODEL TO EXPLORE THE PROCESS OF NEUROGENESIS

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One of the most remarkable forms of structural plasticity in the adult brain is the generation of new functional neurons from adult neural precursors cells (NPCs). There is accumulating evidence that neurogenesis in the adult hippocampus contributes to brain physiology and disease, but its precise physiological role remains elusive. Conceptually, this process can be divided into four steps: (i) proliferation; (ii) neuronal fate determination; (iii) survival and maturation of new neurons; and (iv) functional integration of new neurons into the pre-existing neuronal network. Here we describe the development of phenotypic *in vitro* screening assays using human embryonic stem cell derived NPCs as a cellular model to investigate neurogenesis. Using expression profiling of differentiating cells and exposure of NPCs to bioactive small molecules, we can demonstrate that neurogenesis relevant signaling pathways are active in this *in vitro* cell model. These experiments demonstrate that this human NPC model represents a neuronal relevant transcriptional and biological state, which can unleash the identification of a plethora of targets spanning the druggable genome. Thus, unbiased High-Throughput Screenings (HTS) in combination with image based High Content Analysis (HCA) may represent a powerful tool to identify new active CNS compounds/targets in regenerative medicine, which may ultimately elucidate novel mechanisms modulating adult neurogenesis.

Poster Board Number: 1254

DISRUPTION OF AUTOPHAGY IN A HUMAN NEURONAL MODEL OF NIEMANN PICK TYPE C

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Niemann Pick type C (NPC1) is an incurable, progressive and invariably fatal pediatric dementia caused by mutations of NPC1, a lysosomal membrane protein with a putative role in cholesterol transport. The disease is of tremendous basic science and public health interest due to the intriguing parallels in cellular pathology it shares with Alzheimer's disease (AD), suggesting a common underlying mechanism. Our knowledge of the mechanisms leading to neuronal dysfunction in NPC1 is limited by the difficulty of obtaining live human neurons from affected children. To overcome this difficulty we generated neurons with decreased function of NPC1 from HUES9 human embryonic stem cells (hESC). NPC1 knockdown neurons replicate hallmark phenotypes of NPC1 with accumulation of cholesterol and an abnormal pattern of lysosomal staining. Furthermore, we have described a new phenotype of mitochondrial accumulation not previously reported in NPC1. We tested the hypothesis that abnormal cholesterol processing leads to deficient clearance of mitochondria by the autophagic pathway. Contrary to prior reports, we found that NPC1 fibroblasts and neurons not only have increased activation of autophagy, but also abnormal autophagic flow leading to accumulation of partially degraded mitochondria. These abnormal phenotypes were rescued by treatment with the autophagy inhibitor 3-methyladenine (3MA), and by amelioration of lysosomal cholesterol accumulation with cyclodextrin. Our data suggest a previously unrecognized connection between abnormal cholesterol processing, autophagy defects, and mitochondrial accumulation in NPC1, and reveals a potential new target for the development of new therapies.

Poster Board Number: 1256

NEURAL STEM CELLS FROM DENTAL PAPILLA TISSUE OF HUMAN IMMATURE THIRD MOLAR TOOTH

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INTORODUCTION: In developmental biology, it is well known that tooth development is mediated by the interaction between neural crest-derived mesenchyme and epithelial. During tooth development, nerve is also developed within the pulp tissue through root accompanied with the development of enamel, pulp and ligament structure. Recently, various dental stem cells have been identified in dental tissues such as pulp, ligament, follicle and papilla, and their multi-differentiation including neural differentiation was reported. Regarding the developmental process of dental tissue, the origin of neural crest-derived ectomesenchyme and the neural differentiation activity

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of dental stem cell, it could be hypothesized that neural stem cell can reside in immature soft dental tissue such as papilla tissue. In this study, therefore, the presence of neural stem cells was characterized in the histological section of the human third molar teeth, and neural stem cells were isolated and expanded, and neural differentiation of the isolated neural stem cells was studied. MATERIALS AND METHODS: Neural stem cells were isolated from human dental papilla tissues (KHUSD IRB# 0908-01) by anti-human PS-NCAM antibody and MACS, and characterized by immunocytochemical staining, DNA microarray analysis, flow cytometric analysis and real time-PCR. Neural differentiation and dopaminergic neuronal differentiation was evaluated under proper culture conditions and characterized. RESULTS AND DISCUSSION: A specific cell population expressing neural stem cell markers such as nestin and PS-NCAM was identified within dental papilla tissues of third molar teeth by immunocytochemial staining. During passage of the isolated dental papilla tissue-derived cells, a certain cell population with round morphology demonstrated the characteristics of neural stem cell by expressing several neural stem cell markers; CD133, Nestin, Sox-2, and Sox-9. The neural differentiation of those neural stem cells was induced under a differentiation culture condition containing neurotrophic factors such as NGF, β-mercaptoethanol, laminin and etc. Their neural differentiation activity was also confirmed in coculture with human astrocytes by the positive expression of βIII tubulin and GFAP. Interestingly, at passage 4, approximately 23 % of cell population was PS-NCAM positive. Dopaminergic neurons could be generated from the PS-NCAM positive cells in the presence of shh and FGF 8. CONCLUSION: This research showed the isolation of neural stem cells from dental papilla tissues of human third molar tooth and their neural differentiation capacity including differentiation to dopaminergic neuron, suggesting that dental papillar tissue can be used as a neural stem cell source for stem cell biology and cell therapy. ACKNOWLEDGEMENT: This research was supported by grant No. 20090065530 and 20100003398 from the Basic Research Program of the Korea Science & Engineering Foundation, and also by the National Agenda Project from Korea Research Council of Fundamental Science and Technology.

Poster Board Number: 1258

MAGNETIC ENRICHMENT OF TRA-1-60+ HUMAN PLURIPOTENT STEM CELLS FACILITATES NEURAL SPECIFICATION

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Human pluripotent stem cells constitute an invaluable source for the generation of defined cell types, which may be used for basic research, drug compound screening or ultimatively, regenerative medicine. The specification of neural cell lineages has become relatively convenient after replacing traditional embryoid body/neurosphere induction paradigms with 2D differentiation protocols, which are based on inhibition of SMAD signaling. These protocols give rise to PAX6+ multipotential neuroepithelial (NE) cells as well as AP2+ neural crest (NC) cells within 5-10 days after induction. However, as intermediate step, pluripotent stem cells that are cultivated on mouse embryonic feeder cells are usually transfered to Matrigel and expanded in conditioned medium (CM) before neural differentiation is initiated. Quality and density of the cells in the Matrigel culture are critical parameters that bias the preferential differentiation fate towards NE or NC cells. In order to optimize neural differentation towards either cell type, we have developed a magnetic selection protocol to standardize quality and cell number of truly pluripotent stem cells selected for neural differentiation. Our newly developed method uses magnetic enrichment of TRA-1-60 positive pluripotent stem cells that are immediately transfered to neural induction conditions. At the same time, defined cell densities can be adjusted in order to control differentiation fate towards NE or NC cells. The magnetic enrichment therefore circumvents the need for pre-expansion of pluripotent stem cells in less defined CM conditions before neural induction, saving 3 days of preparation, and more importantly, leads to predictable differentiation results using defined and homogeneous cell populations.

Poster Board Number: 1260

DISC1 REGULATES SURVIVAL OF HUMAN BRAIN DERIVED NEURAL PROGENITOR CELLS VIA MODULATION OF GSK3β/β-CATENIN SIGNALING

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Disrupted-in-schizophrenia 1 (DISC1) is a risk factor for major mental illness, including schizophrenia. We and others have implicated DISC1 in key neurodevelopmental processes including neuronal and neural progenitor cell (NPC) migration, dendrite formation and axon termination. In this study we have further examined the role of DISC1 in human neurogenesis using established human subventricular zone (SVZ) and hippocampal (HP) derived NPC lines with constitutive expression of DISC1 targeting shRNA. DISC1 loss-of-function caused a decreased SVZ- and HP-NPC number and concomitant reduction in the diameter of neurospheres compared to controls. The effect of DISC1 appears to be mediated by activation of GSK3β with ensuing down-regulation of active β-catenin. This is consistent with DISC1 loss-off-function increasing caspase-3/7 activity in SVZ- and HP-NPCs. Importantly, inhibition of GSK3β normalizes NPC number and neurosphere diameter caused by DISC1 loss-of-function. Collectively, our observations implicate DISC1 in cell survival via GSK3β, canonical Wnt/β-catenin signaling, and mitochondrial-mediated intrinsic apoptosis. The results provide a framework for understanding how aberrant DISC1 signaling may contribute to the etiology of psychiatric disorders.

Poster Board Number: 1262

IDENTIFICATION OF CD81'S FUNCTION IN HUMAN NEURAL STEM CELL MIGRATION

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The tetraspanin superfamily (or TM4SF) includes CD81, CD9, CD37, CD53, CD63, CD82 and CD151. Tetraspanin proteins have been implicated in many cellular activities, including adhesion, migration, and proliferation, however their exact function is unknown. Specifically, CD81 (TAPA-1) has been involved in cell migration. But mechanisms are poorly defined in any of stem cells. We describe a novel mechanism whereby the CD81 can affect cell motility and induce migration onto ECM substrates in HNSC. RT-PCR and Western-blot analysis confirmed CD81 expression in HNSC. Immunoprecipitation analysis confirmed CD81 interactions with MMP-9 and integrin beta 1 in HNSC. Also, the expression of the CD81, MMP-9 and integrin beta1 correlated with the HNSC migration. These results demonstrate that CD81 is key receptor in HNSC migration. The shRNA-mediated CD81 downregulation effectively reduced MMP-9 binding to the cell surface, which inhibited the migration of HNSC. Also, Western-blot results show that CD81 stimulates the activity of ERK/MAPKinase in HNSC. Linked with this event, we observed an increase in CD81-associated phosphatidylinositol 3 - kinases activity and mTOR. Our results demonstrate that CD81 as a cell surface binding partner for MMP-9, regulating cell migration and adhesion via intergrin beta1 modulation of tetraspanin/integrin signaling complex. Also, it is well established that CD81 play key roles in cell migration in HNSC cell migration.

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DIRECT GENERATION OF NEUROSPHERE-LIKE CELLS FROM HUMAN DERMAL FIBROBLASTS

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Neural stem cell (NSC) transplantation replaces damaged brain cells and provides disease-modifying effects in many neurological disorders. However, there has been no efficient way to obtain autologous NSCs in patients. Given that ectopic factors can reprogram somatic cells to be pluripotent, we attempted to generate human NSC-like cells by reprograming human fibroblasts. Fibroblasts were transfected with NSC line-derived cellular extracts and grown in neurosphere culture conditions. The cells were then analyzed for NSC characteristics, including neurosphere formation, gene expression patterns, and ability to differentiate. The obtained induced neurosphere-like cells (iNS), which formed daughter neurospheres after serial passaging, expressed neural stem cell markers, and had demethylated SOX2 regulatory regions, all characteristics of human NSCs. The iNS had gene expression patterns that were a combination of the patterns of NSCs and fibroblasts, but they could be differentiated to express neuroglial markers and neuronal sodium channels. These results show for the first time that iNS can be directly generated from human fibroblasts. Further studies on their application in neurological diseases are warranted.

Poster Board Number: 1266

A CELL-FREE EXTRACT FROM HUMAN ADIPOSE STEM CELLS PROTECTS MICE AGAINST EPILEPSY

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Purpose: Stem cell-based therapies are being considered for various neurological diseases, such as epilepsy. Recent studies have suggested that some effects of transplanted stem cells are due to bystander effects that modulate the host environment, rather than direct effects of cell replacement. The extract from human adipose stem cells (ASCs) secreting multiple growth factors including cytokines and chemokines may be a potential source of bystander effects for the treatment of epilepsy, in which inflammation is thought to play an important role. Here, we investigated the effects of a cytosolic extract of ASCs (ASCs-E) in a mouse model of epilepsy. Methods: ASCs-E, boiled ASCs-E, or fibroblast-extract (fibroblast-E) was intraperitoneally administrated to C57BL/6 mice 15 min before pilocarpine-induced status epilepticus (SE) or during chronic epileptic stage. Blood-brain barrier (BBB) leakage was evaluated by measuring Evans blue dye extravasation. Spontaneous recurrent seizure (SRS) was investigated by long-term video-EEG monitoring. The mice performed elevated plus maze, open-field, light/dark transition, and novel object recognition tasks. Results: Acute application of ASCs-E before SE led to earlier attenuation of seizure spike activities after treatment with diazepam, reduction of BBB leakage, and inhibition of the development of epilepsy. ASCs-E treatment (for 7 days) during the chronic epileptic stage suppressed SRS and reduced abnormal epileptic behavioral phenotypes. However, neither boiled ASCs-E nor fibroblast-E had any effects in the experimental epilepsy model. Conclusions: Our results demonstrate that ASCs-E prevents or inhibits epileptogenesis and SRS in mice. They also suggest a stem cell-based, noninvasive therapy for the treatment of epilepsy.

Poster Board Number: 1268

HISTOLOGICAL PATTERNS OF SURVIVAL, FATE AND MIGRATION IN THE INJURED SPINAL CORD AFTER TRANSPLANTATION OF HUMAN NEURAL STEM CELLS AT DOSES RANGING FROM 10,000 TO 500,000 CELLS

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We have previously shown that human central nervous system derived stem cells (hCNS-SCs) promote locomotor recovery after spinal cord injury (SCI) by integrating into host tissue, and that the majority of the hCNS-SCs differentiated into oligodendrocytes when 75,000 cells were transplanted into the spinal cord 9 or 30 days post-SCI. In these studies, the total number of surviving cells was positively correlated with improvements in functional recovery. However, the capacity of the injured spinal cord niche to accommodate donor cells and provide sites and cues for cell differentiation and/or integration may be limited. In this study, we investigated the association between cell dose, i.e. the number of transplanted hCNS-SCs, and engraftment, survival, or differentiation by transplanting 10,000, 100,000, 250,000, or 500,000 cells into the spinal cords of immunodeficient NOD-scid mice after moderate (50kD) mid-thoracic (T9) contusion SCI at an early chronic stage 30 days post-injury. At 16 weeks post-transplantation, unbiased stereological quantification of hCNS-SCs using a human-specific cytoplasmic marker (SC121) demonstrated that the number of engrafted cells was positively correlated with the transplanted cell dose in each group (Pearson $r = 0.79$, two-tailed $p < 0.0001$); that is, a linear relationship was observed between initial transplant dose and the estimated total number of surviving cells determined at 16 weeks post-transplantation. Engraftment and survival was observed across all dose cohorts, however the ratio of surviving cells relative to the initial cell dose was noted to be significantly higher in the low dose group when compared to the other dose cohorts (1-way ANOVA, Tukey's test $p < 0.05$). hCNS-SCs transplanted at all doses showed normal migration from the injection sites into the parenchyma. Quantification of hCNS-SC differentiation revealed that the majority of cells differentiated into SC121+/Olig2+ oligodendrocytes/oligoprogenitors, regardless of the dose group, and that the total number of SC121+/Olig2+ cells was greater in higher dose than lower dose groups. However, the proportion of SC121+/Olig2+ cells varied from 82%7 in the 10,000 cell dose group, to 56%4 in the 500,000 cell dose group when normalized to the total number of surviving SC121+ cells. Statistical analysis revealed that the number of engrafted SC121+ cells and SC121+/Olig2+ cells exhibited a negative correlation (Pearson $r = -0.62$, two-tailed $p = 0.002$). In contrast, no correlation was found between the number of engrafted SC121+ cells and number of either SC121+/DCX+ neural progenitors (7%1.2) or SC123+ human astrocytes (151.5), suggesting the effect of cell dose on fate was lineage specific. Additionally, the number of SC121+ cells not labeled by any of these 3 lineage markers exhibited a positive correlation with the number of engrafted SC121+ cells (Pearson $r = 0.6$, two-tailed $p = 0.003$), suggesting increasing cell dose may alter the kinetics of differentiation. No aberrant histological findings were observed in any transplanted spinal cords, regardless of dose. These preliminary data suggest a possible correlation between cell dose and/or fate/differentiation kinetics in the injured spinal cord, but overall, similar patterns of survival, fate, and migration were observed in the injured spinal cord at transplantation doses ranging from 10,000 to 500,000 cells.

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DIFFERENTIATION OF HUMAN PARTHENOGENETIC STEM CELLS INTO NEURAL STEM CELLS *IN VITRO*

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Unfertilized human oocytes can be artificially activated by appropriate chemical stimuli to develop into parthenogenetic blastocysts, the inner cell mass of which can be isolated and expanded as stem cell lines. Human parthenogenetic stem cells (hpSC) are similar to human embryonic stem cells (hESC) in their proliferation capacity and multilineage *in vitro* differentiation. The hpSC can be either heterozygous or homozygous depending on the way the genome forms from only the maternal chromosome set. Homozygous hpSC could be useful as a source of cells for use in transplantations as the set of HLA genes in hpSC may make differentiated derivatives less susceptible to immune rejection. Furthermore, if the HLA type is common, differentiated derivatives will match many millions of individuals. In addition to these immunogenetic advantages, as parthenogenesis does not involve the destruction of a viable human embryo, the use of hpSC does not raise the same ethical concerns as conventional hESC. Together these two advantages make hpSC a very promising and potentially unlimited source of numerous differentiated somatic cell lines including multipotent neural stem cells (NSC). In this study we derive NSC from hpSC using an approach based on the adherent model with modifications. Primary neural induction was provoked immediately in the colonies of hpSC grown in the absence of feeder cells for 5 days by replacing the culture medium with another one containing bFGF in higher concentration and no serum. After seven days under such conditions rosettes of neuroepithelial cells formed, recapitulating the neural tube of a developing embryo. The rosettes generated in this way expressed specific set of neuroepithelial markers including PAX6, SOX1, NES (Nestin), MSI1 (Musashi-1), and did not express pluripotency marker OCT4 (POU5F1). These rosettes were isolated, disaggregated into single cell suspension and then propagated as an adherent culture. These human parthenogenetic NSC (hpNSC) were passaged every 4-5 days enzymatically with a 1:2 split rate for more than 15 passages. Reverse transcriptase real-time quantitative PCR (qRT-PCR) revealed the expression of specific neural markers NES (Nestin), SOX2 and MSI1 (Musashi-1) in the hpNSC which was also confirmed by immunocytochemical staining of corresponding proteins. The expression of OCT4 was not detected at RNA and protein levels. The cells keep uniform morphology up to at least 15 passages and do not show constantly increasing levels of ectomesenchymal markers SNAI1 and FOXD3 as detected by qRT-PCR. In order to produce neurons and glia cells, we allowed the low density-seeded hpNSC to spontaneously differentiate in culture medium without growth factors for three weeks. After this time most of the differentiated cells have acquired specific neuron morphology and were positive for anti-Tuj1 (Tubulin beta III) labeled antibodies. The expression of neuronal markers TUBB3 (Tubulin beta III) and MAP2 as well as glial markers FOXO4 and GFAP was detected in these differentiated cells by qRT-PCR. Thus, in this study we demonstrated that human multipotent NSC are stable to long-term passaging can be derived from pluripotent hpSC followed by differentiation into neurons and glia.

Poster Board Number: 1272

INTRAARTERIAL DELIVERY OF HUMAN EMBRYONIC-DERIVED NEURAL STEM CELLS YIELDS FUNCTIONAL RECOVERY AND GREATER MYELINATION FOLLOWING HYPOXIC-ISCHEMIC STROKE IN NEONATAL RATS

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Perinatal hypoxic-ischemic insults are a significant cause of neonatal encephalopathy, developmental delays, epilepsy, and cerebral palsy. Studies have shown that demyelination and periventricular leukomalacia are common pathological changes seen in infants following hypoxia-ischemia (HI). While many studies have evaluated the effect of human embryonic-derived neural stem cells (hNSCs) on neurogenesis, angiogenesis and immunomodulation, the impact of hNSC treatment on myelination has not been adequately characterized. Using a neonatal rat model, we investigated the effect of hNSC treatment on remyelination and functional recovery following hypoxia-ischemia. Neonatal Wistar rat pups underwent left common carotid artery ligation followed by placement in a hypoxia chamber with 8% oxygen at 37C on post-natal day 7 (P7). Stroke size was evaluated on P9 using T2-weighted MRI. On P10, the neonates underwent intraarterial injection of either 500,000 fLuc/eGFP transduced hNSCs suspended in 0.9% saline or 0.9% saline only. *In vivo* bioluminescence images utilizing luciferin were obtained 1, 2, 3, 4, 7 and 10 days after injection. BrdU was administered intraperitoneally for 6 days following treatment to assess cell proliferation. Immunohistochemistry stains to evaluate hNSC survival and differentiation were done on stroke-size matched brains in addition to stains for Olig2, NG2 and CNP. Myelination was evaluated using luxol fast blue (LFB) and myelin basic protein (MBP) staining 10 days and 30 days post-treatment. RT-qPCR was performed on hNSCs and the stroked hemisphere of neonates from both treatment groups. Functional recovery was assessed using the elevated open-arm task and novel object recognition task at P30. Bioluminescence imaging demonstrated significant homing of hNSCs to the stroked hemisphere 1 day ($p=0.001$), 2 days ($p=0.002$), 3 days ($p=0.002$), 4 days ($p<0.0001$), and 7 days ($p=0.001$) following transplant. Iba-1-/GFAP-hNPCs were localized to the corpus callosum and cortex of cell-treated animals 3 days, 10 days, and 30 days after treatment. Counts of BrdU+ cells indicated a significant increase in cell proliferation around the lateral ventricle ($p=0.036$) and in the corpus callosum ($p=0.020$) of cell-treated animals compared to saline-treated animals. Cell-treated neonates also showed an increase in Olig2+ and NG2+ cells in the striatum 3 and 10 days post-treatment. LFB and MBP staining demonstrated greater myelination 10 days and 30 days after treatment in the corpus callosum ($p=0.022$, $p=0.049$) and striatum ($p=0.017$, $p=0.001$) of cell-treated animals. Increased expression of mRNA associated with cell proliferation (TGF1, p53, Stat3) and immunomodulation (IL-10, ApoE, Hsp1) were seen in the stroked hemisphere of cell-treated animals. Neonates transplanted with hNPCs demonstrated better performance on the elevated open arm task ($p=0.050$) and novel object recognition task ($p=0.016$). Intraarterial hNSC delivery following hypoxia-ischemia in neonatal rats resulted in early hNSC homing to the injured brain with engraftment seen up to 30 days following treatment. Behavioral performance on the elevated open arm task and novel object recognition task suggest cell-treated animals have reduced levels of anxiety and improved working memory compared to saline-treated animals. Functional recovery may be mediated by improved myelination and stimulation of oligodendrocyte-lineage cell proliferation in the corpus callosum and striatum.

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HUMAN EMBRYONIC STEM CELLS CULTURED ONTO A MEF-DERIVED ORGANIZED MATRIX FORM SYMMETRIC EMBRYOID BODIES PRONE TO THE NEURAL PHENOTYPE

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Human embryonic stem (hES) cells have been exhaustingly cultured on mouse embryonic fibroblast (MEF) feeder layers. Although this approach maintains hES cells pluripotency and proliferative properties for long-term periods, embryoid bodies (EBs) derived from this system are morphologically irregular, varying in size and symmetry. Here we describe a strategy for the formation of symmetric EBs by culturing hES cells on ECM coats without the requirement of MEF feeder layers. This system abolishes feeder-stem cell contact, maintaining the organized characteristic of feeder-produced matrices, which differs from unorganized matrix systems, such as matrigel. ECM was obtained from MEFs by chemical treatment. H9 cells cultured onto plates previously coated with MEF-derived ECM (MEF-ECM) were compared with H9 cells cultured on mitomycin C-inactivated MEFs (control) for 100 days. Cells were analyzed for the expression of pluripotency markers revealing that H9s cultured on either control MEFs or MEF-ECM expressed similar levels of Oct-4, SOX-2, TRA-1-60 and SSEA-4. EBs derived from MEF-ECM H9 cells were smaller (136 ± 32 μm) compared to control embryoid bodies (202 ± 65 μm) and more symmetric, with a longer/shorter diameter ratio closer to 1 (1.14 ± 0.13) when compared to the control group (1.33 ± 0.28). In addition, when stimulated to the neural phenotype, MEF-ECM derived EBs presented higher levels of the neural markers SOX-2 and β-III tubulin compared to the control EBs. In conclusion, we show here that hES cells differentiation dynamics can be altered by changing colony substrate to a MEF-ECM coat, and this alteration leads to a change in EB morphology and specific lineage differentiation.

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FROM HUMAN EMBRYONIC STEM CELLS TO SYMPATHETIC NEURONES: A MODEL FOR UNDERSTANDING NEUROBLASTOMA PATHOGENESIS

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Background and Aims: Neuroblastoma is an embryonal tumour originating from neural crest cells which give rise to the sympathetic nervous system (SNS). Our aim is to differentiate human embryonic stem cells along neural crest and autonomic lineages to sympathetic neurons, providing a model to increase our understanding of the pathogenesis of neural crest derived malignancies, including neuroblastoma, as well as the potential of cell replacement for neurological disease. Results: Using the stromal-derived activity (SDIA) of mouse PA6 we induced differentiation of H9 and Ncl-14 - clinical grade hESC into neural crest and autonomic progenitors. Expression of neural crest specifier genes by RT-PCR including, Snail, Slug, Sox-9, and the noradrenergic marker, dHAND were increased at days 7 and 14 of PA6 co-culture, subsequently decreasing by day 21. Following 21 days of co-culture extensive neuronal differentiation was observed by Immunofluorescence with high expression of the neuronal markers NCAM, Phox2b, peripherin and tyrosine hydroxylase. The addition of BMP4 (5nM) at days 5-9 of co-culture led to an increase in Peripherin expression by RT-PCR. FACS sorting for the NC stem cell marker p75 enriched for cells expressing p75, peripherin, dopamine beta-hydroxylase and tyrosine hydroxylase indicative of sympathetic neuronal differentiation. Conclusions: Our data shows a model of normal SNS development which can be used to understand more about neural crest pathogenesis.

Poster Board Number: 1278

FUNCTIONAL ASSAYS IN HUMAN EMBRYONIC STEM CELL DERIVED NEURAL CREST CELLS TO DETECT NEURODEVELOPMENTAL TOXICANTS

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Proper neural development consists of several tightly regulated processes including for example cell differentiation, neurite outgrowth and migration. The latter has a particularly important role in the development of the PNS. Neural crest cells (NCC) emerge from the neural tube and migrate throughout the body along distinct paths to give rise to multiple cell types including neural and non-neural cells. To study potentially adverse effects of different compounds on the migratory potential of neural precursors, we used human embryonic stem cells (hESC) differentiated into NCC. We were able to keep the cells in a neural crest progenitor state, which allowed the expansion and freezing of the cells. Furthermore, the NCC could be differentiated into peripheral neurons and Schwann cells, which confirmed their functional potential, while marker expression indicated the expected phenotype. To study the migratory potential of these cells and the effects of compounds on the migration capacity, we used the classical scratch assay which has been used successfully in many studies in different laboratories. A scratch was created within the confluent monolayer of NCC and the cell migration was analyzed in the presence and absence of compounds known to inhibit cell migration. Various negative controls and known developmental neurotoxicants like e.g. CH3HgCl were tested. LIVE cell imaging experiments demonstrated that the cells repopulate the scratch via migration, independent of cell division. We also showed that compounds which inhibit actin polymerization, inhibit the migration of these cells at concentrations that do not affect general cell viability or cell division, while negative control compounds had no effect on the migratory potential of the cells. In addition to the classical scratch assay and manual counting, we used actin-GFP transfected cells to investigate the effects of compounds on the actin cytoskeleton in real time using LIVE cell imaging. A second very important aspect in neurodevelopment is neurite outgrowth. Once the neural progenitor cells have reached their correct destination after the migration phase, proper establishment of the neurite network is essential for full functionality of the neurons. We found that the NCC, once differentiation was initiated, start forming multiple projections, which later in the differentiation process develop into neurites, which are at least 3 times longer than the diameter of the cell. The formation of these initial cell processes was decreased upon treatment with the potent protein kinase C inhibitor bisindolylmaleimide 1, in a concentration dependent manner, without affecting general viability of the cells. The system presented here is suitable for high throughput, high content image analysis which allows the investigation of effects of different chemicals on the migration of NCC and on the initial process formation, leading to neurite outgrowth of NCC differentiating into peripheral neurons. We therefore believe that our human based in-vitro test system is a powerful tool to detect potential neurodevelopmental toxicants that affect early events during neural development e.g. migration of NCC and later events like neurite outgrowth of NCC differentiating towards peripheral neurons. As lentiviral transduction of these cells can be achieved with a high efficency (≥ 95%) the system allows

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functional analysis of the mode of action and specific pathways involved in the toxic effects of different chemicals.

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NATURALLY SECRETED AMYLOID β OLIGOMERS DERIVED FROM APPSW EXPRESSEING CELLS DISTURB PROLIFERATION, INDUCE GLIOGENESIS AND ATTENUATE MIGRATION OF HUMAN NEURAL STEM CELLS

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Neural stem cells (NSCs) derived from developing and adult brain are defined by their ability to self-renew, to differentiate into cells of all glial and neuronal lineages throughout the neuraxis, and to populate developing or degenerating CNS regions. Alzheimer's disease (AD) is an inexorable neurodegenerative disease which neuropathologically reveals extracellular accumulations of amyloid beta (Aβ) and intracellular inclusion of hyperphosphorylated tau in the brain. Recent studies suggest that especially, soluble Aβ oligomers have a main deteriorative role in the synaptic and neuronal function of AD. To understand neuronal loss and the cognitive impairment in AD, a number of studies have performed to investigate whether Aβ could regulate the proliferation and differentiation of NSCs. However, it still remains controversial. In order to investigate the effects of Aβ oligomers on NSCs, we prepared Swedish mutated amyloid precursor protein (APPsw) expressing SK-N-MC cells using lentiviral vectors and confirmed that these cells secreted Aβ oligomers into the culture media. Human neural stem/progenitor cells (hNSCs) were isolated from telencephalic tissue of a fetal cadaver at 13 weeks of gestation and cultured as neurospheres. When naturally secreted Aβ oligomers generated from APPsw expressing SK-N-MC cells were directly administered to hNSCs under the proliferative condition, hNSCs showed the decrease in cell division via the induction of a senescence but not apoptosis. Under the differentiation condition, Aβ oligomers secreted from cells remarkably induced gliogenesis of hNSCs at the level of both mRNAs and proteins. In addition, hNSCs little migrated across the membrane of the trans-well in Boyden chamber when hNSCs were co-cultured with APPsw expressing SK-N-MC cells rather than co-cultured with naive cells. The results provide that naturally secreted Aβ oligomers produced from APPsw expressing SK-N-MC cells adversely regulate proliferation, enhance glial differentiation, and debilitate migration of hNSCs *in vitro*. These findings suggest that the pathologic microenvironments of brain in AD may negatively regulate the homeostatic functions of NSCs in CNS which may cause the cognitive impairment and neural dysfunction in AD.

Poster Board Number: 1282

LONG-TERM SURVIVAL AND MIGRATION OF ALLOGENEIC HUMAN CENTRAL NERVOUS SYSTEM STEM CELLS FOLLOWING INTRACEREBRAL TRANSPLANTATION IN NEURONAL CEROID LIPOFUSCINOSIS

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Neuronal Ceroid Lipofuscinoses (NCL), also known as Batten Disease, is a rare and fatal lysosomal storage disorder that primarily affects the central nervous system. The infantile (INCL) and late-infantile (LINCL) subtypes result from inherited mutations that produce defective or deficient palmitoyl-protein thioesterase 1 (PPT1) and tripeptidyl peptidase I (TPP-I) lysosomal enzymes, respectively. *In vivo* cross-correction of an affected host by human central nervous system stem cells (HuCNS-SC®) transplantation formed the rationale for a Phase I clinical trial in which unmodified donor cells supply the deficient lysosomal enzymes. The Phase I dose-escalation trial involved both intracerebral and intraventricular transplantation of purified HuCNS-SC cells into six patients with advanced INCL or LINCL, followed by 12 months of immunosuppression. To date, three patients have expired secondary to natural progression of the disease during either the Phase I study or separate four year long-term follow-up (LTFU) investigation. Post-mortem examination of the entire brain from all three patients was conducted to assess for adverse histological changes as well as the presence of donor cell engraftment. One female patient with INCL in the low-dose cohort, still receiving immunosuppression, expired 11 months post-transplant during the Phase I study. Two other patients, both with LINCL, expired during the ongoing LTFU investigation; one male patient in the low-dose cohort at 2.5 years post-transplant and one male patient in the high-dose cohort at 3.5 years post-transplant (each received 12 months of immunosuppression as per protocol). Post-mortem examination revealed expected disease-related changes in all patients. No evidence of cellular atypia, cyst formation, neoplasm, or inflammatory reaction to HuCNS-SC transplantation was found. Evidence of donor cell engraftment was assessed by differences in genomic DNA sequences between the donor cells and the recipient by PCR-based analysis. The results show that HuCNS-SC can engraft and survive in the NCL host well after immunosuppression has been discontinued. Additional details regarding donor cell quantification and migration in individual patients will be presented. This report is the first to demonstrate long-term survival of a purified population of allogeneic neural stem cells following human transplantation. The postmortem results showing no adverse histological changes combined with HuCNS-SC survival supports allogeneic cell therapy for neurological disorders.

Poster Board Number: 1284

TRANSCRIPTIONAL PROFILING OF COFACTOR PROTEINS BY REST/NRSF IN NEURONAL STEM CELL USING PROTEOMICS ANALYSIS

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Repressor element-1 silencing transcription factor/neuron-restrictive factor (REST/NESF) is known to be a major transcriptional repressor of neurogeneous. For REST/NRSF-dependent promoter repression to occur, REST/NRSF must interact with several cellular cofactors, including Co-REST, N-CoR, mSin3A, and the histon deacetylase complex. However, despite the variety of interesting findings turned up thus far cofactors for the tight regulation of REST/NRSF activity remain to be identified. In present study, we have investigated to identify of a cofactors binding in around REST/NRSF in F3 human neuronal stem cell. Using monoclonal anti NRSF and gold shell with magnetic nanobeads pull -down assay, 2-D electrophoresis and LC/mass spectrophotometry analysis, a total 20 specific binding proteins showed significant change in their expression in F3 cells. These proteins were categorized by their function, those included transcription factor and metabolism related protein. To support the proteomic results, the presence of three candidate proteins, HIS1, HIS2 and HIS3, were further characterized in this study. The binding of these proteins with the NRSF were verified using chromatinimmunoprecipitation and immunoprecipitation analysis. These data suggest that these specifically interacting cofactors may play an important role in neuron cell specific expression of the neuronal stem cell. Also, these newly identified NRSF binding proteins provide a number of clues and potential links to understanding the mechanisms regulating the transcription of neurogenesis.

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HUMAN AND MOUSE NEURAL STEM CELL CHEMOKINE RECEPTOR AND LIGAND EXPRESSION DIFFERENCES AND THEIR IMPLICATIONS FOR NEUROREGENERATIVE MEDICINE

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Introduction: Recent studies demonstrate the potential of intravascular stem cell therapies for the treatment of many CNS disorders, including stroke. Success of intravascular stem cell therapies hinges on neural stem cell (NSC) targeted homing and subsequent intraparenchymal migration to injured area, processes reliant on NSC expression of chemokines and their receptors. As these therapies come closer to clinical trials, it is essential to understand the interspecies differences of chemotaxis related receptor and ligand expression to expedite the transition from rodent research to human application. Here we use RT-qPCR to compare human neural stem cell (hNSC) and mouse neural stem cell (mNSC) chemokine receptor and ligand expression at basal levels. Furthermore, we compare hNSCs and mNSCs after exposure to MCP-1 to test the response of NSCs to a chemokine shown to be upregulated in the microenvironment of the ischemic brain. Finally, we explore the implication of these differences on cell migration. Methods: Human and mouse cells were grown as monolayers in their respective growth media. In pretreated cell groups, MCP-1 (20ng/mL) was introduced to cell media 1 hour prior to RNA extraction. RT-qPCR was performed on extracted RNA using microarray technology for chemokine receptors and ligands. The Boyden chamber migration assay was used to determine migration to SDF-1 at concentrations 0ng/mL, 100ng/mL, 500ng/mL, 1000ng/mL, and 1500ng/mL. Results: Analysis of baseline expression demonstrated that 18% of analyzed factors were different between hNPCs and mNPCs ($p<0.001$). The five factors with the largest fold regulation difference between hNSCs and mNSCs are CCL2, CCL7, CXCL12, CMLKR1, and CXCR4. After treatment with MCP-1 there was a 1.61% change in factor expression between human and mouse chemokine. Of the mRNA transcripts upregulated above 3 fold regulation, ligands accounted for 6 of 6 factors in mNPCs and 1 of 1 in hNPCs. Of the mRNA transcripts downregulated below -3 fold regulation, receptors accounted for 5 of 5 factors in mNPCs and 1 of 3 in hNPCs. Migration of hNSCs compared to mNSCs in response to SDF-1 was greater by 2.46 fold ($p< 0.01$), consistent with the greater expression of CXCR4 on hNSCs than mNSCs. Conclusion: Baseline chemokine receptor and ligand expression between human and mouse NSCs was significantly different, possibly resulting in interspecies differences in NSC response to the ischemic brain microenvironment. In the context of cell therapy for stroke, the implications of interspecies distinctions should be strongly considered when conclusions drawn from rodent research are translated to human treatment.

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ENRICHMENT OF MURINE NEURAL STEM CELLS FOR TRANSPLANTATION IN LYSOSOMAL STORAGE DISEASE MODEL

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Neural stem cells (NSCs) are potential vehicles for delivery of therapeutic agents in the diseased CNS. Our lab has previously shown that it is possible to deliver a therapeutic agent directly to the mouse brain by transplantation of immortalized C17.2 NSCs, which integrate into the CNS in a cytoarchitecturally appropriate manner. While C17.2 and similar clonal cell lines have been very useful for certain experiments, they were selected for robust engraftment properties, and thus are not representative of the heterogeneous populations of autologous NSCs that would be used for *ex vivo* gene therapy. The biology of C17.2 cells also differs significantly from primary neural stem cells, particularly in that primary murine NSCs exhibit much more limited migration ability. Since only a limited number of cells can be transplanted into the brain, enrichment for the most 'stem-like' cells in the donor population may increase engraftment. To test this, we selected NSCs at an early stem cell stage using the cell surface marker CD15 (Lewis X). A 'gentle FACS' procedure (low sheath pressure, larger nozzle size, and low events/sec) was used to increase post-sorting viability and yield. Approximately 6% of adult mouse SVZ cells were CD15+ and selection resulted in a 15-fold enrichment. The phenotype was relatively stable in culture, with 70% remaining CD15+ after 10 passages. The sorted cells differentiated into the three major neural lineages (oligodendrocytes, astrocytes and neurons). Although more donor cells were seen in transplants of enriched cells than with unsorted cells, they still exhibited limited migration. Cells were detected near the lateral ventricles, in the cortex, in the perimeter of the hippocampus and in the region surrounding third ventricle. We also analyzed the CD15+ population in the SVZ of a mouse model of a neurogenetic disease, the lysosomal storage disorder mucopolysaccharidosis type VII (MPS VII). Recent work from our lab on canine MPS VII NSCs and on the transcriptome of normal vs MPS VII mouse brain regions, suggest that developmental defects are present in MPS VII NSCs and are influenced by the diseased microenvironment. Significantly more CD15+ cells were present in the forebrain of MPS VII mice than unaffected controls at two months of age ($p<0.026$) which increased further by four months ($p<0.0143$). The MPS VII mouse brain has progressively increasing numbers of degenerating neurons with age, thus the corresponding increase in NSCs in the diseased brain may be due to a compensatory response to replace depleted mature cells.

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A NOVEL LONG-LIVED IMMUNODEFICIENT MOUSE MODEL OF MUCOPOLYSACCHARIDOSES TYPE 1 SUITABLE FOR PRECLINICAL TRIALS OF STEM CELL COMBINATORIAL THERAPY

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Mucopolysaccharidosis type 1 (MPS-1, Hurler Syndrome), a lysosomal storage disorder (LSD), is an inherited deficiency of the lysosomal glycosaminoglycan (GAG)-degrading enzyme α-L-iduronidase (IDUA) in which GAG accumulation causes progressive multi-system organ dysfunction, neurological impairment, and death. Current MPS-1 mouse models, based on a NOD/SCID (NS) immunodeficient background, are short-lived, providing a very narrow window to assess the long-term efficacy of therapeutic intervention. These mice also develop thymic lymphomas, making assessment of potential tumorigenicity of human stem cell transplantation problematic. Neural stem cell (NSC) transplantation, in combination with hematopoietic stem cell (HSC) transplantation, may provide a robust treatment for these patients by providing enzyme to the central nervous system (CNS) as well as the periphery. We therefore developed a new MPS-1 model by breeding MPS-1 NS mice with the recently developed NOD/SCID/Il2rγ (NSG) mouse.

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This model is long-lived and tumor-free, and engrafts significantly better than the NS-based model. NSG MPS-1 mice exhibit the phenotypic features of MPS-1 including coarsened fur and facial features, reduced/abnormal gait, kyphosis, and corneal clouding. We examined tissue IDUA and GAG accumulation, skeletal abnormalities, tissue pathology, and engraftment of human neural stem cells (NSCs) and human HSCs in this new MPS-1 model. IDUA was undetectable in all tissues examined while GAG levels were dramatically higher in most tissues. MPS-1 brain showed a significant inflammatory response and prominent gliosis compared to normal brain. NSCs and HSCs were found to readily engraft in this long-lived mouse model with human cells detectable for at least one year post-transplantation. This new model, therefore, is suitable for preclinical testing of combination stem cell therapies aimed at providing multimodal (peripheral and CNS) treatment for LSDs such as MPS-1 and provides a platform to move novel stem cell therapies toward Phase 1 clinical trials.

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EXPANSION OF MURINE EMBRYONIC CORTICAL STEM CELLS USING DOXYCYCLINE INDUCIBLE N-MYC EXPRESSION

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Large-scale source of neural stem cells hold great potential for the treatment of disorders involving extensive cellular damage like spinal-cord injuries and also for other neurodegenerative disorders like Parkinson's disease & Ischemic cerebral stroke. Besides its therapeutic potential, such cells would also serve as ex-vivo model for studying complex molecular pathways governing neural cell fate choices. Here we report the establishment of a novel transgenic system utilizing doxycycline inducible N-myc expression that allows for a million-fold expansion of cortical stem/progenitors isolated from E12.5 mouse embryos within a span of 25 days in culture. When these cells are propagated in presence of doxycycline, N-myc expression is induced and they undergo rapid proliferation while maintaining the expression of neural stem cell markers. After prolonged *in vitro* expansion, upon doxycycline withdrawal from the culture media, N-myc expression is turned off and the cells differentiate normally into neurons and glia. The ability of expanded progenitors to form neurons even after 25 days of culture as opposed to wild-type cells that generally lose the neurogenic potential within 6-8 days, suggests that doxycycline induced N-myc expression effectively 'locks' the cells in an undifferentiated state and aids rapid proliferation by triggering the cell-cycle progression. Furthermore, normal diploid G-band karyotype confirms the chromosomal stability and genomic integrity of the expanded cells. Currently engraftment assays involving transplantation of these cells into the brain of adult mice is being performed to determine their in-vivo differentiation potential into various neural cell types. Furthermore, we also performed differential gene expression analysis between the N-myc induced and the non-induced cell populations to determine the genetic networks that get activated in response to N-myc expression.

Poster Board Number: 1294

FUNCTIONAL IMPROVEMENT OF CEREBELLAR DEGENERATION AFTER INTRA-CEREBELLAR TRANSPLANTATION OF HUMAN FETAL NEURAL STEM CELLS INTO SPINOCEREBELLAR ATAXIA TYPE 3 MICE

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Introduction: Spinocerebellar ataxia types 1, 2, 3, 6, and 7 are examples of polyglutamine diseases which are caused by an abnormal expansion of repetitive CAG bases, which encode for the amino acid, glutamine. In particular, spinocerebellar ataxia type 3 (SCA3) which is associated with beyond 50 glutamine repeats, is characterised by progressive neuronal degeneration, cerebellar ataxia and variable findings associated with dystonic-rigid extrapyramidal syndrome, peripheral neuropathy and/ or amyotrophy. Employing SCA3 mice as a model of cerebellar degeneration, we hypothesised that intracerebellar human fetal neural stem cell (hfNSC) transplantation will result in improvement of the clinical phenotype. Methods: Multipotent regionally-derived cerebellum (C) and subventricular zone (S) hfNSC (15 week gestation) were cultured as neurospheres (NS). Xenogeneic transplantation of 105 C-hfNSC (n=6) and S-hfNSC (n=8) were injected into the cerebellum of P22-P28 SCA3 mice under cyclosporine immunosuppression. At D8 and D53 post injection, rotorod studies at accelerating speed of 0 to 40RPM in 3min as well as steady speeds of 5 and 7RPM were performed. Mice were euthanised at both time-points for patch clamp analysis of electrophysiological properties. Results: Transplantation of S and C-hfNSC resulted in an improvement in accelerating rotarod latency (time taken to fall) of 26.9% and 38.5% over controls at D8. Improvements of 21.6% and 28.2% over controls at D53 were observed for steady-speed rotarod latency at 5RPM. Improvements of 32.7% and 24.0% were observed over controls at D8 and 0% and 51.1% at D53, respectively when the SCA3 mice were subjected to rotarod turning at 7RPM. Multi-innervation of Purkinje cells by climbing fibres indicates the receipt of both proper and improper signals by the Purkinje cells and patch-clamp analysis conducted at D8 demonstrated a threefold improvement in reduction of multi-innervated Purkinje cells; from 60% in the control to 20% in hfNSC injected SCA3 mice. However, these differences were transient and were not observed at D56 time-point (ratio of 40%, 57.1% and 44.4%, respectively). Conclusion: Our data suggest functional improvement of cerebellar degeneration in SCA3 mice post xenogeneic hfNSC transplantation, alluding to the utility of hfNSC for the treatment of polyglutamine-related neurodegenerative disorders. Further optimisation of the cell source, dosage, monitoring outcomes and therapeutic window may be required to ascertain the true efficacy of intra-cerebellar transplantation of hfNSC into SCA3 mice.

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NOVEL ROLES OF WNT/β-CATENIN SIGNALING IN MURINE CORTICAL NEURAL PRECURSOR FATE AND LAMINAR IDENTITY CONTROL REVEALED IN THE ABSENCE OF ADHESION DEFECTS

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The size and the complexity of the developing brain are determined by the exquisite balance between self-renewal and differentiation of neural stem cells (NSC). Among multiple signaling cascades active in the developing forebrain, the canonical Wnt pathway was shown to regulate NSC proliferation, fate choice and dorso-ventral specification. The transducer of Wnt signals, β-catenin, interacts with TCF/LEF family members, but it also participates in adherens junctions as a bridging component. Thus, experimental overexpression and downregulation of β-catenin cannot distinguish between its signaling and adhesion functions. Combining a dorsal forebrain conditional inactivation approach with different β-catenin mutant alleles, we identify here the contribution of β-catenin transcriptional activity as opposed to adhesion in the development of the cerebral cortex. In agreement with previous reports, Wnt/β-catenin signaling ablation causes premature NSC cell cycle exit, which leads to microcephaly in the absence of ectopic BMP signaling. Similarly, inactivation of canonical Wnt signaling at the midline leads to a shortened hippocampal primordium and a dorsolaterally shifted hem. Contrary to published data, however, we do not observe a role of Wnt signaling in the dorso-ventral patterning of neural precursors and proper cortical layering, but, interestingly, in the control of laminar identity and sequential fate switch by NSCs.

Poster Board Number: 1298

NEUROPROTECTION OF GRAFTED OLIGODENDROCYTE PROGENITORS IN A MOUSE MODEL OF EXCITOTOXICITY

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Background: One of the major problems of the premature neonate is the extensive white matter loss that is a major cause of childhood neurological disability, especially, periventricular leukomalacia (PVL). PVL is now recognized as the most important cause of cerebral palsy and mental retardation occurring at a high incidence in preterm (<32 gestational weeks) babies. Various studies have shown that in PVL lack of healthy OL leads to myelin deficiency and ventriculomegaly (enlargement of ventricles) conditions. When a premature infant is born, clinicians take preventive precautions to insure survival but, a potential treatment to reduce or prevent white matter loss does not exist at the present time. In the developing brain, oligodendrocytes (OL) maturation occurs perinatally, and immature OL are particularly vulnerable to insults. Myelinogenesis and myelin maintenance depend on the presence of healthy OL in adequate numbers. We previously identified a specific combination of two trophic factors, namely TS1 that promoted endogenous OL regeneration and CNS remyelination in the postnatal, as well as, the adult rodent brain. We recently described that a single injection of two trophic factors, namely TS1 partially alleviates white matter injury (WMI) in a mouse model of glutamate excitotoxicity (GME), by conferring neuroprotection to endogenous nestin-expressing progenitors and OL progenitors present in the brain at the time of the insult. TS1 also stimulated the generation of new OL progenitors that were able synthetize myelin reducing the enormous white matter loss found in control mice. Because endogenous neural progenitors might not be enough to overcome vetriculomegaly, cell replacement therapies may need to be used to enhance the extent of recovery of white matter in PVL. Thus, here we investigated if grafted OL progenitors would survive in the excytotoxic brain to further myelinate CNS axons. Methods: The periventricular white matter lesion was produced by a single injection of NMDA into the floor of the CC above the lateral ventricle (LV). Five conditions were studied in a comparative manner: Saline, NMDA, NMDA+TS1 and NMDA+TS1+OLP and NMDA+OLP were stereotaxically injected simultaneously into p4 mouse pups. For ventricular measurements, the area of ipsilateral "IL" vs. contralateral "CL" ventricles was compared. These values were also compared against saline injection. Brains were examined at various time points (7 to 35 days) after treatment. Each treatment was performed in triplicates. Cell populations were evaluated for neural commitment or uncommitted by the expression of specific OL markers and nestin. Cell proliferation was evaluated with the antibody Ki67. Results: Out of all treatments a reduction of ventriculomegaly was found in the presence of TS1 with an increased myelination with respect to mice treated with NMDA alone. Moreover, in the absence of TS1, OLP did not survive the excitotoxic insult while in mice that received NMDA+TS1+OLP, grafted cells survived and migrated to white matter areas. Conclusion: PVL appears to be treatable, both by neuroprotection of endogenous progenitors, as well as with a combinatorial therapy in which cell grafts and trophic factors would be administered together to counteract the effects of excitotoxicity. This work was funded in part by a pilot grant from the American Multiple Sclerosis Society and by NIHD-0657 grant.

Poster Board Number: 1300

HIGH EFFICIENCY MYELINATION OF THE HYPOMYELINATED SHIVERER MOUSE BRAIN USING HUMAN IPS CELL-DERIVED GLIAL PROGENITOR CELLS

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We have previously described techniques for the identification, isolation, molecular characterization, and transplantation of glial progenitor cells derived from the fetal human brain. Neonatal engraftment by these cells permits the widespread myelination of congenitally dysmyelinated shiverer mouse brain, and can rescue both the neurological phenotype and lifespan of these animals. Yet the possibility of immune rejection of allogeneic grafts has hindered the clinical development of this approach. To address this issue, we investigated the use of human induced pluripotent stem cells (hiPS cells) as a source from which new glial progenitor cells (GPCs) and their derived oligodendrocytes (OLs) might be generated. We developed a high-efficiency strategy by which to differentiate hiPS cells into GPCs, that are in turn able to mature into both astrocytes and myelinogenic OLs, *in vitro* and *in vivo*. Using this protocol, we have successfully generated and validated a population of olig2+/PDGFRα+/Nxk2.2+/sox10+ human GPCs. Using this protocol, we found that the efficiencies of GPC induction from hiPSCs (KØ4 cells, K. Hochedlinger, keratinocyte-derived; C27 cells, L. Studer, fibroblast-derived) were similar to that of human embryonic stem cells (hESCs; WA09), in that their otherwise unsorted yields of olig2+/Nkx2.2+GPC colonies were 63% (KØ4) and 79% (hES WA09), respectively. hiPSC-derived GPCs, as well as their oligodendrocytic and astrocytic progeny, could then

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be differentially isolated using FACS directed at glial lineage markers that included A2B5, CD140a/ PDGFRα, CD9, O4, and GLT1. In addition, these hiPSC-derived GPCs could be differentiated into mature MBP+ OLs and GFAP+ astrocytes in culture. Importantly, these hiPS-GPCs readily matured into myelinogenic oligodendrocytes *in vivo*, and efficiently myelinated the forebrains of myelin-deficient shiverer mice by 12-13 weeks after neonatal graft. Of note, no tumor formation was noted from these grafts as long as 3 months after transplant. These results demonstrate that high efficiency *in vivo* oligodendrocytic differentiation and myelination can be achieved from human iPS cells, suggesting the potential utility of iPS-derived autografts in treating acquired disorders of myelin.

Poster Board Number: 2002

DIRECT INDUCTION OF SAFE NEURAL STEM CELLS FROM ADULT MOUSE FIBROBLASTS

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Induced pluripotent stem (iPS) cells give rise to neural stem cells (NSCs), which are applicable for therapeutic transplantation in treatment of neural diseases. However, generation of patient-specific NSCs from iPS cells requires a careful selection of safe iPS clones without teratoma formation. This precludes autograft transplantation for acute neural injuries which requires cell transplantation before their conditions are chronically fixed. We sought to determine whether direct induction of NSCs from partially reprogrammed somatic cells is able to generate safer cells rapidly. After 14 days of serum-free culture of mouse fibroblasts partially reprogrammed by several retrovial vectors including Oct3/4, they generated mature neurospheres which give rise to neurons, astrocytes, and oligodendrocytes while primary neurospheres derived from ES and iPS cells generated mostly neurons. Further, by optimizing culture conditions, these neurospheres contained Nanog-positive cells less than 0.01%. To evaluate the safety and engraftment of these neurospheres, they were transplanted into mouse striatum. Teratomas were observed in less than 10% of transplanted animals while more than 80% of clonal-iPS-derived neurospheres induced from adult mouse fibroblasts generated teratomas. These results suggest that NSCs derived from partially reprogrammed fibroblasts can differentiate more rapidly compared to ES/iPS-derived NSCs. We propose that the direct induction without generation of iPS clones facilitiate quick and safe induction of NSCs for cell therapy.

Poster Board Number: 2004

MOUSE NEURAL PRECURSOR CELLS EXPRESS TROPHINS FOLLOWING TRANSPLANTATION BUT REMYELINATION IS THE KEY MECHANISM BY WHICH THEY AUGMENT FUNCTIONAL RECOVERY FOLLOWING RODENT SPINAL CORD INJURY

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Background: Though myelination has been demonstrated following neural precursor cell (NPC) transplantation, the mechanism(s) by which NPCs enhance functional recovery from spinal cord injury (SCI) is unclear. NPCs have also been postulated to elaborate trophins following transplantation which could lead to tissue sparing or other beneficial effects. Methods: Two weeks following clip compression SCI at the T7 bony level, rats underwent cellular or control transplantation. Transplants included wild-type mouse NPCs, NPCs derived from shiverer mice unable to produce central myelin, freeze-thaw killed dead NPCs or cell suspension media. In most experimental groups animals also received minocycline, cyclosporin and peri-lesional infusion of trophins via an osmotic mini-pump. Hind-leg motor function was assessed weekly for 6 additional weeks according to the BBB scale. qPCR for trophin expression was performed on NPCs and host cord isolated by FACS sorting 1w post-transplant. Hematoxylin-eosin/luxol fast blue staining was used to assess grey and white matter, cyst and lesional tissue. Axonal preservation was assessed using Fluorogold retrograde tracing. As well, tissue bearing cells was stained for the axonal marker NF200 and the myelin marker MBP. Transplanted cells expressed the eYFP gene facilitating their identification. Confocal microscopy was used to assess the relationship between transplanted cells and axons, as well as their ability to make MBP. Results: Animals transplanted with wild-type NPCs showed significantly greater functional recovery than any other group. Animals transplanted with shiverer NPCs performed worse on functional testing than even injured and untreated animals, suggesting harm. Confocal microscopy confirmed that wild-type NPCs ensheath axons and form MBP. Shiverer NPCs also appeared to ensheath axons but did not produce MBP. NPCs isolated post-transplant expressed all four neurotrophins, CNTF, EGF, and bFGF at higher levels than host tissue. Our transplantation protocol is associated with grey, white matter and oligodendrocyte sparing however this was attributable to pharmacotherapy and trophin infusion. Trophin infusion was associated with an increase in cyst and lesional tissue volume as well as inflammatory cell numbers and reduced functional recovery. No experimental therapy led to preservation of axons. Discussion: Our results suggest that remyelination by NPCs plays a dominant role in enhancing functional recovery. Shiverer NPCs may prevent remyelination by endogenous cells capable of myelin formation leading to harm. The mere presence of transplanted cells did not lead to a functional benefit suggesting that the trophins they produce are without a functional effect. Indeed, the tissue sparing seen in our protocol is attributable instead to the pharmacotherapy and trophin infusion. The trophin pump, with its tip 1cm rostral to the epicenter, was associated with undesirable histological changes and impaired functional recovery. Conclusions: These results suggest that remyelination by NPCs is their most important contributor to functional recovery following SCI. Though NPC transplantation may have other effects, they do not appear to contribute to functional recovery in our transplantation paradigm. Though these findings may be specific to our protocol, these findings may lead to strategies to augment functional recovery associated with cellular transplantation for SCI.

Poster Board Number: 2006

MCL-1 PROMOTES NEURAL PRECURSOR CELL CYCLE EXIT AND DIFFERENTIATION IN THE MOUSE EMBRYONIC BRAIN

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Neural precursor cell (NPC) proliferation and apoptosis are key regulatory aspects of mammalian nervous system development. Although recent studies suggested these two processes to be interrelated, the molecular mechanisms behind this remain undefined. Here we show that myeloid cell leukemia-1 (Mcl-1), a Bcl-2 family member that is essential for the survival of NPCs also causes premature terminal mitosis in NPCs. *In utero* electroporation of Mcl-1 in E13.5 mouse embryonic brains show that within 48 hours, the majority of NPCs transfected with Mcl-1 have migrated into the post mitotic cortical plate, whereas control transfected NPCs are still within the proliferating ventricular/subventricular zones. Analysis of proliferation by proliferating cell nuclear antigen (PCNA) immunohistochemistry reveal a 2-fold reduction in proliferating NPCs in the Mcl-1 treated brains. Immunohistochemistry for Tbr1, a marker for newborn neurons, reveal a 50%

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increase in differentiated neurons in Mcl1 treated brains. BrdU birthdating demonstrates that Mcl-1 overexpression results in a greater cohort of newborn neurons. Mcl-1 transfected NPCs gave rise to neurons in deeper layers of the cortex than control transfected NPCs confirming an earlier birthdate. Similarly, transfection of Mcl-1 in NPCs *in vitro*, promotes cell cycle exit. Finally, we found an increase in Cdk inhibitor p27kip1 protein, a key promoter of cell cycle exit with Mcl-1 overexpression and a concomitant decrease in p27kip1 in Mcl-1 conditional knockout NPCs, suggesting that Mcl-1 may stabilize p27kip1 protein to promote NPC differentiation. In summary, these results identify a novel function for Mcl-1 in promoting terminal mitosis of NPCs by influencing the cell cycle regulatory machinery.

Poster Board Number: 2008

DERIVATION OF HIGH PURITY OLIGODENDROCYTE PROGENITOR CELLS FROM MOUSE EMBRYONIC STEM CELLS

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Mouse embryonic stem cells (mESCs) demonstrate remarkable proliferative and developmental capacities. The derivation of mESC lines from normal and transgenic mice, including non-viable breeds, and their subsequent differentiation into high purity cell populations would permit novel developmental studies, screening assays and transplant-based research. Here we present a novel method for obtaining oligodendrocyte progenitor cells (OPCs) in high purity from mESCs. Our differentiation protocol benefits from a decade of developmental stem cell research and underscores important differences in culturing methods such as plating surfaces, media composition and culturing duration that can influence derivation purity. Overall, this differentiation protocol provides a means of generating high purity rodent OPCs for use in studies of oligodendroglial lineage development, screening assays for compounds that affect the oligodendroglial lineage, and OPC replacement strategies. Indeed, we are currently using this protocol for the differentiation of OPCs from mutant shiverer and wild-type matched mESCs for future use in various rodent-based diseased models.

Poster Board Number: 2010

DYSTROGLYCAN REGULATES THE ORGANIZATION AND GLIOGENIC CAPACITY OF THE MOUSE NEURAL STEM CELL NICHE

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The subventricular zone (SVZ) is a specialized microenvironment surrounding the lateral ventricles of the vertebrate forebrain. Arising perinatally from the neuroepithelium, the SVZ niche harbors neural stem and progenitor cells (NSCs/NPCs) that produce glia and olfactory interneurons throughout life. Recently, investigations into the architecture and cellular arrangement of the adult SVZ have demonstrated the importance of extracellular matrix (ECM) proteins in the maintenance of the niche. In particular, activated stem cells are often found in close proximity to laminin-rich blood vessel basal laminar and fractone-associated ECM structures. Our preliminary observations indicate that an organized laminin-rich SVZ ECM has been established by birth and may have a role in regulating the principal wave of gliogenesis that occurs within the first two postnatal weeks. The laminin receptor dystroglycan (DAG), with established roles in adhesion, ECM reorganization, cellular polarity and proliferation, is a good candidate receptor for the transduction of laminin signaling in SVZ cells. Here, we tested the possibility that dystroglycan has a role in early postnatal subventricular zone architecture, proliferation and gliogenesis. We utilized the Cre/loxP approach to generate mice in which dystroglycan is deleted at the neural stem cell stage (DAG CNS KO). Dystroglycan-deficient radial glia, the resident stem cells at birth, appear disorganized and fail to form apical endfoot associations with laminin structures at the ventricular surface. Normally, wild-type Sox2+ neural stem and progenitor cells become less proliferative in the first few postnatal days, however DAG KO Sox2+ cells retain their earlier proliferative capacity. DAG KO Sox2+ nuclei are also found in an aberrant distribution, such that they proliferate closer to the ventricular surface than WT Sox2+ cells. The corpus callosa of early postnatal DAG KO mice contain more oligodendrocyte precursor cells (OPCs) than those of WT littermates, with a higher proportion of OPCs co-expressing the NSC markers Nestin and Sox2, as well as fewer mature oligodendrocytes. Together these findings suggest that loss of dystroglycan contributes to a developmental delay in oligodendrocyte lineage cells. In support of this, neurosphere differentiation assays reveal that NPCs isolated from DAG KO mice produce more OPCs but fewer post-mitotic and myelinating oligodendrocytes. *In vivo*, this delay in oligodendrocyte differentiation in turn leads to delayed myelination of the corpus callosum. In summary, we report that dystroglycan-laminin interactions in the perinatal 'gliogenic' SVZ are important for stem and/or progenitor cell organization and gliogenic capacity. Ongoing studies are addressing if and how dystroglycan-mediated cytoskeletal reorganization contributes to the rate and symmetry of neural stem and progenitor cell division to maintain the fine balance between self-renewal of the neural stem cell niche and the production of more lineage-restricted progenitors.

Poster Board Number: 2012

ADULT RAT MESENCHYMAL STEM CELLS DERIVED SOLUBLE FACTORS PRIME PROLIFERATING NEURAL PROGENITORS INTO AN OLIGODENDROCYTE FATE

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Oligodendrogenesis encompasses lineage specification of neural progenitors (NPCs), differentiation into oligodendrocytes, which ultimately culminates in the myelination of central nervous system axons. Each individual process must be tightly regulated by extracellular and cell-intrinsic mechanisms, whose identities are barely known. We had previously demonstrated that soluble factors derived from rat mesenchymal stem cells (MSCs) induce oligodendrogenesis in differentiating adult NPCs. However, since lineage specification typically occurs in proliferating progenitors and not during differentiation, we investigated if soluble factors derived from MSCs are able to prime proliferating NPCs (as neurospheres) to the oligodendroglial fate. Therefore, we analyzed the effects of conditioned media derived from MSCs (MSC-CM) on adult rat derived neurosphere cell morphology, proliferation, cell-specific marker expression profile, response to growth factor withdrawal, and the expression of astroglial and oligodendroglial fate determinants. While MSC-CM did not affect the proliferation rate of NPCs, it induced the formation of cellular processes and adherence of neurosphere cells. Even though the expression profile of the progenitor and precursor markers Nestin, GFAP, A2B5, NG2, O4, and DCX in neurosphere cells was not affected by MSC-CM, the formation of MBP positive oligodendrocytes after growth factor withdrawal was boosted by the MSC-CM pre-treatment. Moreover, in proliferating neurospheres, the presence of MSC-CM reduced the anti-oligodendrogenic determinant Id2, thus increasing the relative proportion of the pro-oligodendrogenic factor Olig2 expression. Finally, when MSC-CM pre-treated neurospheres were exposed to an astrogenic milieu, the proportion of MBP-expressing oligodendrocytes was increased compared to non pre-treated neurospheres, indicating that the MSC-CM

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priming effect involved a strong oligodendrocyte lineage commitment. In summary, soluble factors derived from MSCs prime proliferating progenitors towards oligodendrogenic fate. The present findings underscore the potential use of MSCs in cell therapies for remyelination such as in multiple sclerosis and spinal cord injury. Moreover, they urge the identification of the oligodendrogenic activity(ies) derived from MSCs in order to develop novel molecular therapies for demyelinating diseases.

Poster Board Number: 2014

EPIDERMAL GROWTH FACTOR STIMULATION OF ADULT RAT NEURAL STEM CELLS REVEALS UNIQUE CELLULAR PHENOTYPES, STRUCTURAL REORGANIZATION AND NEOANGIOGENESIS

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Objective: To study the ultrastructural and phenotypic characteristics of Epidermal Growth Factor (EGF) stimulated adult rat neural stem cells and subventricular zone neurogenic niche in the context of early tumor development. Background: The highly proliferative neural stem cell population in the brain, which shows several glial characteristics, could be a likely source of glial tumors in both the immature and adult CNS. Brain tumors are the most common type of solid tumor in children and are very hard to treat due to the inherent risk of damaging healthy brain tissue and normal brain function. The activity of several growth factors, important for neural stem cell proliferation, has been demonstrated to be altered in both experimental models and clinical cases of brain tumors. Epidermal growth factor (EGF) is a potent mitogen with widespread effects on neural stem cells. It is known to stimulate proliferation and inhibit neuronal maturation of neural stem cells. The specific effects of EGF on the fate determination of rat neural stem cells *in vivo* is poorly studied, in spite of EGF being widely used to stimulate stem cell proliferation *in vitro*. Upon EGF infusion, profound structural reorganization is observed and hyperplasias are formed from the subventricular zone. The hyperplasias show certain unique characteristics not seen in the control SVZ, both in terms of cellular phenotype and the surrounding niche. Results: Apart from confirming previous results, showing the retention of EGF stimulated cells in a stem cell/glial like state, we characterize, in detail, the cell population stimulated by EGF infusion. A large subset of the EGF stimulated proliferating cells is expressing Sox2 or Olig2, with a significant portion of these cells being co-labeled, not observed in the control SVZ. Other markers such as GFAP, nestin, vimentin, NG2, and DCX were also analyzed. We show an increase in stem cell activity in the lateral ventricle walls upon EGF stimulation; inducing hyperplasia formation enriched for stem cells and halting normal differentiation from early stem cell stages. We observe structural reorganization, breakdown of the ependymal cell layer, and absence of junctional complexes in hyperproliferative zones. Occasionally, these zones exhibit signs of hypoxia, microglial activation, and neoangiogenesis. Characterizing the EGF-responsive stem cell population and elucidating its role in neoplastic growth and restructuring of the subventricular zone neurogenic niche could prove useful in increasing the understanding in early stages of tumor development and cancer transformation in neural stem cells.

Poster Board Number: 2016

THE ANTI-COAGULANT FACTOR PROTEIN S AND ITS STRUCTURAL HOMOLOG GAS6 REGULATE NEURAL STEM CELL PROLIFERATION IN THE SUBVENTRICULAR ZONE OF ADULT RODENTS

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Vitamin K-dependent proteins (VKDP) are a family of secreted proteins mainly known for their role in coagulation. VKDP share a post-translational modification catalyzed by an enzyme that requires vitamin K, necessary for their secretion and/or activity. VKDP production is blocked by warfarin, a vitamin K antagonist that is a widely used anticoagulant. Recently, two vitamin K-dependent proteins, Gas-6 and protein S, have identified as ligands for the TAM tyrosine kinase receptors (Tyro 3, Axl, Mer) which regulate normal development and tumorigenesis, especially in the brain. Given the emerging pleioptropic functions of VKDP and TAM receptors, we assessed possible regulatory roles of these mechanisms over proliferation and stemness of neural stem cells in the adult mammalian brain. We focused our study on a major brain germinative center, the subventricular zone (SVZ) bordering the lateral ventricles. We demonstrate that the suppression of functional VKDP production, *in vitro*, by exposure of subventricular zone (SVZ) neural stem cell cultures to warfarin or *in vivo* by its intracerebroventricular injection to mice, leads to a substantial increase in SVZ neural stem cells proliferation which is reversed by either vitamin K or endogenous VKDP, suggesting that VKDP constitutively inhibit neural stem cells proliferation. We further identify protein S and Gas6 as the two only VKDP produced by SVZ cells. We demonstrate that the anti-coagulant factor protein S inhibits while neutralization of endogenously produced protein S enhances SVZ cells proliferation, implying that protein S is a constitutive inhibitor of SVZ cells proliferation. Further, our data show that in Gas6 knock-out mice, loss of Gas-6 reduces SVZ neural stem cells pool. By identifying protein S and Gas6 as novel regulators of neural stem cells, our study opens new insights in the regulation of stem cells activity by vascular determinants and suggests new perspectives for the pharmacological use of the anti-coagulant warfarin.

Poster Board Number: 2018

A ROLE FOR THE REDOX/FYN/C-CBL PATHWAY IN MODULATING OXIDANT-INDUCED CELL CYCLE ARREST IN RODENT OLIGODENDROCYTE PRECURSOR CELLS

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Proper myelination of the central nervous system requires the tightly regulated time- and region-specific generation of oligodendrocytes from precursor cells, a process that involves timely cell cycle exit and the execution of a differentiation program. Previous work from our laboratory has identified a molecular pathway – termed the Redox/Fyn/c-Cbl pathway – that integrates environmental cues and the balance between differentiation and self-renewal in rodent oligodendrocyte/type II astrocyte (O-2A) progenitor cells, a biological decision which we have previously demonstrated is regulated by

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the intracellular redox state. The present work describes a role for this pathway in modulating cell cycle exit in response to oxidizing, pro-differentiation signals in O-2A progenitor cells. We also describe the involvement of this pathway in modulating cell cycle exit induced by chemical pro-oxidants that appear to mimic the anti-mitotic effects of physiological, pro-differentiation signaling molecules.

Poster Board Number: 2020

STEM CELL TRANSPLANTATION STRATEGIES FOR REPAIR OF THE DYSMYELINATED CNS

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Neural stem and progenitor cells (NSPCs) show great potential for cellular replacement in models of cortical and sub-cortical injury and dysmyelination. NSPCs derived from the sub-ventricular zone of yellow fluorescent protein transgenic animals and grown in spheres were injected bilaterally into the anterior and posterior aspects of the anlagen of the corpus callosum and also into the anlagen of the cerebellar peduncle. Animals were injected, using a pulled glass needle, on P0, P7 (equivalent to human birth) and P21 (equivalent to ~2 years human age) in a shiverer mouse model of dysmyelination and survived for 4 days, 14 days or 45 days. These cells integrated both anatomically and functionally into the local white matter, as well as the periventricular areas. Transplanted aNPCs did not become GFAP+ astrocytes, but preferentially became MBP+, Olig2+ oligodendrocytes that interacted with NF200+ axons. Trans callosal stimulation showed that MBP+ transplanted cells were capable of restoring a fast action potential, corresponding to the presence of functionally myelinated fibres, in the corpus callosum of transplanted shiverer animals. This evidence indicates that aNPCs are capable of restoring myelin conduction in brain white matter tracts, thereby illustrating their translational potential for other CNS demyelinating or dysmyelinating diseases.

Poster Board Number: 2022

LABEL FREE ISOLATION OF NEURONAL AND GLIAL PROGENITORS

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Generation of differentiated neurons and astrocytes from neural stem/progenitor cells (NSPCs) of the developing cerebral cortex occurs via the production of progenitors biased to either neuronal or glial fates. Despite developmental studies documenting these progenitors, a precise description of the cellular characteristics that discriminate them is lacking, partly due to limitations in available markers for each specific cell type. We have previously shown dielectrophoresis (DEP), which is a label free technique for characterizing cells, distinguishes both human and mouse neuronal progenitors from their glial counterparts. Progenitor cell dielectric properties shift as the cells lose neurogenic potential over time in culture and a specific measure of the membrane properties of the cells, membrane capacitance, is clearly linked to cell fate potential. DEP has been used successfully in other contexts to sort distinct cell populations and our studies have shown exposure to DEP forces necessary for cell sorting does not harm human or mouse NSPCs. We therefore used DEP frequencies to isolate cells from a mixed population of NSPCs and found that cells isolated at lower frequencies generated astrocytes while those collected at higher frequencies formed neurons. These results show that dielectric properties define specific progenitor cell populations and these properties can be used for their isolation. Specific dielectric properties can be added to the list of characteristics that distinguish neurogenic from gliogenic progenitors and isolation of these progenitors by DEP can provide purified populations of cells for further analysis and determination of discrete cellular properties.

Poster Board Number: 2024

SYNDECAN-1 IN THE FOREBRAIN GERMINAL NICHE INSTRUCTS THE APICAL TO BASAL PROGENITOR TRANSITION BY INHIBITING THE NOTCH PATHWAY

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Two major classes of dorsal neural progenitor cells contribute to mammalian cerebral cortical neurogenesis. Apical cells in the ventricular zone (VZ) are the principle progenitor cells for the cortex. They directly generate neurons and also produce a secondary set of transit amplifying precursors, the basal progenitor cells, that move up to the sub ventricular zone (SVZ). The mechanisms underlying lineage progression from apical to basal fates are still largely unknown, and in particular, the environmental regulators that stimulate this important transition are being actively sought. This is an important topic because it has relevance to stem cell-transit amplifying transitions in other tissues. Syndecan-1 is a transmembrane proteoglycan involved in cell-matrix interactions and it influences the activity of a number of different signaling pathways. The extracellular domain of Syndecan-1 can be cleaved and the shed form of Syndecan-1 is biological active. Here we report that Syndecan-1 is highly expressed in the germinal zone of the developing cortex. In cultured forebrain cortical progenitor cells, we found that Syndecan-1 is necessary for production of basal progenitor cells, acting both cell and non-cell autonomously. In the embryonic cortex *in vivo*, Syndecan-1 knockdown via *in utero* electroporation leads to depletion of both apical and basal progenitor cells, and to premature neuronal differentiation, while over-expression increases progenitor proliferation. We found that over-expression of Syndecan-1 promotes the switch from apical to basal progenitor cell fates and disorganizes the VZ/SVZ structure. The extracellular integrin-binding domain, amino acids 67-121, is essential for the over-expression phenotype. In analysis of how Syndecan-1 elicits the apical-basal transition, we discovered that exogenous Syndecan-1 inhibits Notch signaling and up-regulates Neurogenin-2 leading to increased expression of Tbr2, a key determinant of the basal progenitor fate. Thus Syndecan-1, which is expressed by both progenitor cells and endothelial cells in the ventricular zone niche, is a critical environmental factor capable of instructing stem cells to change to the transit amplifying state. As Syndecan-1 is present in other germinal zones and is up-regulated in several tumors, it will be important to examine whether it plays a similar role in this pathological context.

Poster Board Number: 2026

TIGHT REGULATION OF TRANSGENE EXPRESSION IN NEURAL PROGENITOR CELLS WITH A MIFEPRISTONE-INDUCIBLE SELF-INACTIVATING LENTIVIRAL DELIVERY SYSTEM

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An important tool for studying stem cells is the ability to regulate expression of target genes. We have developed a mifepristone (MFP) inducible system for regulated expression of target genes in neural progenitor cells (NPCs). Potential uses include *in vitro* or *in vivo* applications for gene/cell therapy. As MFP readily penetrates the blood-brain barrier, this system is particularly amendable for use in the CNS. Components of the system were packaged into self-inactivating (SIN) lentiviral vectors for *ex vivo* delivery to NPCs.

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Several different target transgenes including fluorescent markers, antibiotic resistance genes, and therapeutic proteins were placed into these vectors and specific strategies were used for selecting NPCs with robust MFP-inducible transgene expression with minimal background. Our system consists of two separate components, a Switch vector and a Target vector. Several variants of each of these vectors were produced. Switch vectors express a chimeric nuclear receptor (SWITCH) consisting of the GAL4 DNA-binding domain, an NFkB transactivation domain, and a truncated progesterone receptor ligand-binding domain. When activated by MFP, the SWITCH protein binds to GAL4 response elements (UAS) and initiates transcription of downstream target transgenes. Autoinducible SWITCH vectors were produced using promoters consisting of the rat nestin enhancer linked to UAS sequences and a thymidine kinase minimal (TK) promoter. These nestin promoters were active in murine and human NPCs (and HEK293T cells). Internal ribosome entry sites were used to link SWITCH expression to selectable markers such as the hygromycin-resistance or eGFP genes. Target vectors were developed with different inducible promoters and target genes. MFP-inducible promoters contained UAS sequences linked to a TK promoter. Numerous target loci were created using combinations of antibiotic resistance genes, MdsRED, eGFP, luciferase, receptor tyrosine kinases (RTKs), and/or neurotrophins. Specific selective strategies utilizing FACS or antibiotic selection were used to enrich for cells with robust MFP-inducible target transgene expression and minimal background expression. Switch and Target vectors were delivered to cells in a stepwise manner. Murine and human cells were infected with Switch vectors first and selected by antibiotic resistance or FACS. SWITCH expression was robust and was downregulated upon NPC differentiation. Switch-expressing cells were then infected with Target vectors containing selectable markers. Dual-infected cells were exposed to MFP or vehicle. Initially, the fidelity and inducibility of the system was evaluated using fluorescent marker target transgenes. In the absence of MFP there was no expression of these transgenes; while MFP exposure induced robust expression. Other Target loci with transgenes encoding receptor tyrosine kinases or neurotrophins linked to antibiotic resistance genes also showed very tight control of transgene expression *in vitro*. *In vivo* analysis of the MFP-inducible NPC system is ongoing. Rodents will undergo CNS transplantation with NPCs capable of inducible expression of target transgenes. Initial analysis will look at NPCs inducibly expressing fluorescent markers and/or luciferase. Rodents will be treated with placebo or MFP for set periods of time and expression of target transgenes will be evaluated by fluorescent microscopy, immunohistochemistry, or bioluminescence.

Poster Board Number: 2028

DIFFERENTIAL REGULATION OF RETINOIC ACID SIGNALING IN ADULT SPINAL CORD NEURAL PROGENITORS

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Neural stem cells from the adult subventricular zone (SVZ) are highly heterogeneous, with their position of origin being a key factor in determining the neuronal subtype they can give rise to. Whether this diversity extends to other regions in the adult CNS has not been demonstrated. *In vitro* studies with directed neuronal differentiation of ES cells suggest that subtype specification may be regulated by the intrinsic positional identity of the ES-derived cell, since altering the positional identity leads to corresponding changes in motor neuron subtype. This limited plasticity suggests the position identity of the original stem cell source is a critical factor for the generation of the desired neuronal subtype. The adult spinal cord consists of endogenous stem/progenitor cells which are activated upon injury, with potential for repair in CNS diseases and spinal cord injury. However, our knowledge and understanding of these cells are limited. Our research is aimed at understanding the properties of endogenous progenitor cells in the adult spinal cord and how they can be utilized for neuronal regeneration. In this study, we identified multiple subpopulations of spinal cord progenitor cells (SCPCs) based on their position along the anterior/posterior (A/P) axis of the adult spinal cord. These subpopulations can be distinguished by the expression of distinct combinatorial Hox genes in a manner reminiscent of their expression in the developing neural tube. Moreover, different progenitor subpopulations display varying cellular properties, such as a higher neurogenic potential and higher neurosphere-forming ability observed in lumbar-derived progenitor cells. We further demonstrate that axial-derived SCPCs are differentially responsive to the neurogenic agent retinoic acid (RA). In the presence of RA, neurogenesis was increased by two-fold during differentiation of cervical-derived spheres, while no increase was observed for lumbar-derived cells. Expression profile analysis of RA signaling components revealed that the RA degrading enzyme cyp26b1, absent in cervical SCPCs but highly expressed in lumbar SCPCs, is likely to regulate RA signaling in these SCPC populations. Our study suggests that different niche factor regimens are required for site-specific neuronal regeneration of endogenous SCPCs from distinct spatial regions.

Poster Board Number: 2030

TRANSPLANTATINON OF NEURAL STEM CELLS IMPROVES OUTCOME FROM SPINAL CORD INJURY IN AGED MICE

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Neural stem cells (NSCs) transplantation have a role in restoring locomotor function after spinal cord injury (SCI), but how aging might affect the spinal cord's receptivity to such transplants is unknown. In the present study, we compared the effect of NSCs transplantation for SCI in between young adult (2-month-old) and aged (15-month-old) mice. After inducing contusive SCI by IH impactor (70kDyn), NSCs derived from CAG-ffluc-venus Tg mice were transplanted into the injured spinal cords 9 days after SCI. Behavioral analysis using BMS score and bioimaging of the grafted cells were performed until 6 weeks after SCI. The aged mice showed severer neurological deficit compared to the young adult mice in consistence with the larger lesion site and less spared myelination area after SCI. The aged mice, however, exhibited the higher survival rates of NSCs compared to the young adult mice, resulting in similar functional recovery to the young adult mice. RT-RCR of the injured spinal cords at 2 weeks after SCI revealed a lower gene expression of sema3a and higher gene expression of HGF in the aged mice, suggesting that the favorable microenvironment of the injured spinal cord in the aged mice might support the survival of the grafted NSCs and the axonal regrowth. Taken together, advanced age did not reduce beneficial effects of NSCs transplantation for SCI.

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Poster Board Number: 2032

A NONVIRAL IPSC MODEL FOR THE STUDY OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is the most common age-associated dementia. In these studies we generated nonviral, nonintegrated human induced pluripotent stem cell (hiPSC) lines for *in vitro* and *in vivo* modeling of neurons and glial cells harboring specific AD mutations. This AD stem cell model provides unique opportunities for diagnostic and drug testing strategies. We describe the derivation of AD hiPSC lines derived with nonintegrated episomal constructs from fibroblasts of patients carrying familial AD mutations, as well as from healthy age-matched control fibroblasts. The two AD patient fibroblast donors included one 56-year old male and one 56-year old female, both carrying the missense mutation Ala246Glu (A246E) in the presenilin 1 (PS1) gene (Catalog No.AG06840 and AG06848; Coriell Institute for Medical Research, Camden, NJ). PS1 mutations are the most common cause of early-onset familial AD. Reprogramming was achieved with nonintegrating oriP/EBNA1-based episomal vectors for the transient expression of OCT4, SOX2, KLF4, LIN28, NANOG, c-Myc and SV40LT transgenes. AD-affected hiPSC were characterized for their pluripotency by teratoma analysis, cytology, expression of human ES markers, karyotype analysis, and methylation/demethylation profiling. hiPSC from AD and healthy subjects were differentiated into neural cells with a Noggin induction protocol to demonstrate AD pathogenesis, including differential expression and cleavage of APP, and impairment of neurogenesis and neurite outgrowth. Neuronal differentiation of AD-PS1 precursors was characterized via qRT-PCR and immunocytochemical procedures for neuroepithelial/neural markers (e.g., Pax6, Sox1, nestin, musashi) as well as neuronal markers at various stages of maturation (doublecortin, βIII tubulin [TUJ1], MAP-2). Neuronal differentiation of PS1/A246E precursors was measured with semiquantitative ICC and flow cytometry and was compared to precursors from control hESC. We also examined the differential expression of APP and the levels of Aβ40 and Aβ42 in differentiated hiPSC using quantitative anti-Aβ enzyme-linked immunosorbent assay (ELISA) from AD-derived neurons and controls. These studies provide a valuable foundation for characterizing the pathologic development of AD neural precursors.

Poster Board Number: 2034

NEURONAL DIFFERENTIATION OF NON-VIRAL DOWN SYNDROME IPS CELLS REVEALS KNOWN AND NOVEL DISEASE PHENOTYPES

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Down syndrome (DS, trisomy 21) affects both pre- and post-natal brain development and neuronal function. Because Down syndrome research has heavily relied on cells derived from 20 week old fetal brains or mouse model systems little is known about the effect of trisomy 21 on early human brain development. We therefore generated DS and control hiPS cells from primary fibroblasts using episomal vectors and, following extensive characterization, differentiated these cells into neurons and glial cells. We show that although DS iPS and control iPS transcriptomes are highly similar and closely resemble human embryonic stem cell, DS-hIPS display over-expression of 68 chromosome 21 genes as well as differential expression of 1200 non-chromosome 21 genes. We correlate these expression data with genome wide methylation comparisons between control anbd Down syndrome iPS cells. Upon stepwise neuronal differentiation of DS IPS over-expression of additional chromosome 21 genes, such as APP, DYRK1a and SOD1, is observed, revealing for the first time cell type dependent activation of specific chromosome 21 genes in DS. Importantly, neuronal cultures derived from DS iPS derived neurospheres display increased spontaneous and hydrogen peroxide induced cell death, shorter neurite extensions and increased glial differentiation over time in culture, phenotypes previously also observed in primary human fetal derived DS neurons. We however also identify deregulation of Notch and Hedgehog signalling at specific temporal windows of early Down syndrome neurogenesis not previously identified in primary foetal derived neuronal cultures. We conclude that non-viral DS iPS cell derived neurons recapitulate known features of DS brain pathologies but can also be used to identify novel DS associated phenotypes.

Poster Board Number: 2036

PATIENT-SPECIFIC INDUCED NEURONS DIRECTLY FROM REPROGRAMMED FIBROBLASTS FUNCTION IN THE ANIMAL BRAIN

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Regenerative medicine aims to create healthy and functional cells of the same type to replace diseased or damaged tissues. Here, we show that a combination of three transcription factors Ascl1, Myt1l and Sox2 is capable of converting human fibroblasts from the scalp of patients with traumatic brain injury (TBI) to neurons directly. 28 days after gene transduction, fluorescence-activated cell sorting (FACS) was performed using PSA-NCAM, a marker for immature neurons or neuronal progenitors. The percentage of PSA-NCAM+ cells was 7.62%. By further cultured, these sorted cells expressed the pan-neuronal markers Tuj1 and synapsin. To explore whether these cells possessed the characteristics of functional neurons, we analyzed the electrophysiological properties. Patch-clamp recordings demonstrated that, these cells expressed the voltage-gated ion channels, such as fast inactivating inward (Na+-channels) and outward currents (K+-channels). These cells generated spontaneous action potentials as early as 35 days after transduction albeit few spikes may indicate them more like less mature neurons. Three weeks after transplantation into the brain of NOD/SCID mouse, human iN cells were immunostained with markers of various types of neuron. We observed voltage-gated sodium and potassium channels. Both excitatory and inhibitory spontaneous postsynaptic currents (EPSCs and IPSCs) had be detected, suggesting iN cells formed functional postsynaptic compartments and receive synaptic inputs from host neurons. To test the potential for nerve regeneration with iN cells, we evaluated the effectiveness of iN cells transplantation as a treatment for TBI. Adult male NOD/SCID mice received a weight-drop injury as previous method to induce brain trauma. Five days after injury, iN cells were injected into the injured cortex. Open field BBB scoring showed significant differences between iN cells and either human fibroblast or vehicle transplanted controls (n=10, $p<0.05$). These cells were integrated into the mouse brain and promote locomotor function recovery. Generation of iN cells from patient-derived fibroblasts heralds immense hope in the neural regenerative medicine field.

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Poster Board Number: 2038

ABERRANT PURINERGIC SIGNALING IN IPS CELLS DEFICIENT IN HPRT EXPRESSION

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Lesch-Nyhan disease (LND) is an X-linked genetic disorder associated with mutations of the hypoxanthine guanine phosphoribosyltransferase (HPRT) gene leading to purine metabolic abnormalities and neurobehavioral manifestations including dystonia and compulsive self-injurious behavior. Although available evidence has identified a role for purinergic signaling in neuronal differentiation and brain development, mechanisms linking HPRT deficiency, purinergic pathways and neural dysfunction have not been well defined. In this study, we have used lentivirus vectors expressing shRNA targeted to the HPRT gene to produce HPRT-deficient human induced pluripotent stem (iPS) cells. In HPRT knockdown cells, real-time PCR analyses revealed severely decreased mRNA levels of the purinergic P2Y1 receptor compared to control cells and fluorescence-activated cell sorting (FACS) analyses confirmed a decreased cell surface expression of P2Y1. Exposure of cells to the P2Y1 blocker MRS2179 and the P2Y1 agonist ATP revealed dysregulated expression of phosphorylated CREB and ERK1/2 in HPRT knockdown cells. Additional studies of *in vitro* differentiation of normal and HPRT-deficient iPS cells may clarify a role of HPRT in purinergic mechanisms of the central nervous system and in the development of LND and other neurodevelopmental and neurodegenerative diseases.

Poster Board Number: 2040

THE EXPRESSION AND ROLE OF MACROPHAGE MIGRATION INHIBITORY FACTOR IN NEURAL STEM/PROGENITOR CELLS

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In previous studies, we demonstrated that mouse dendritic cells (DCs) can increase the number of neural stem/progenitor cells (NSPCs) *in vitro* and *in vivo*. In this study, we identified macrophage migration inhibitory factor (MIF) that is secreted from DCs and NSPCs as a novel factor that can support the proliferation and/or survival of murine NSPCs *in vitro*, although the function of MIF in the normal brain remains largely unknown. It was previously shown that in macrophages, MIF can bind to a receptor complex of CD74 and CD44. In the present study, we observed a CD74/CD44 double-positive cell population in mouse ganglionic eminence (GE)-derived neurospheres using flow cytometry technique *in vitro*. Interestingly, secretion of MIF from neurospheres was also confirmed by ELISA. We further found the expression of CD74 in GE of E14 mouse brain, suggesting the functional role of MIF *in vivo*. Exogenous addition of MIF to NSPCs increased the number of primary and secondary neurospheres in the presence of EGF and FGF2 and did not affect the differentiation potential of NSPCs. In contrast, retrovirally expressed MIF shRNAi suppressed the secondary neurosphere formations and cell proliferation, and increased the caspase3/7 activity in neurospheres. Moreover, we found that in neurospheres MIF increases the phosphorylation of Akt, Erk, and Stat3 (Ser727) which are known as factors supporting the cell survival, proliferation and/or maintenance of NSPCs. The cell surface expression of GLUT1 was also increased with AMPK activation by MIF treatment in neurospheres, showing that MIF may contribute to the cell proliferation and/or survival through glucose uptake. These results suggest that MIF can cause proliferation and maintain NSPCs utilizing multiple-signaling pathways synergistically in an autocrine manner and may be a new therapeutic factor for neural degenerative disorders through NSPCs activation.

Poster Board Number: 2042

NEURAL STEM CELLS SURVIVE, MIGRATE AND ENGRAFT OPTIMALLY WHEN DELIVERED INTRA-ARTERIALLY AT 3 DAYS AFTER HYPOXIC-ISCHEMIC STROKE

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Intro: Minimally invasive intra-arterial (IA) neural progenitor cell (NPC) transplantation is a promising avenue for stem cell therapy after stroke. However, the effects of timing of transplantation on cell survival remain unknown in this specific model. Methods: Mouse NPC's (5x105 in 5µl saline) harboring a reporter gene construct containing renilla luciferase were delivered to the brain via the carotid artery at 5 distinct time points after hypoxic-ischemic (HI) stroke. These groups were assigned as the 6 hours, 24 hours, 3, 7 and 14 days post-HI transplantation groups. Cell engraftment was monitored by in-vivo bioluminescence imaging (BLI) for luciferase activity, performed 6 hours, 4 days, and 7 days after IA injection. Stroked brain homogenates were analyzed using RT-qPCR for the chemokines CCL2 and SDF-1, and adhesion molecule VCAM-1. In-vitro Boyden Chamber migration and quantitative RT-qPCR was also used to characterize C17.2 NPCs. Results: BLI at 6 hours after transplantation revealed a significantly higher luciferase signal in the 3 days post-HI transplantation group as compared to the 6 hours ($P=0.015$), 7 days ($P=0.015$), and 14 days ($P=0.016$) post-HI transplantation groups. This trend continued at 4 days after transplantation. At 7 days after transplantation, the 3 days post-HI transplantation group was statistically significant to all other groups ($P<0.05$). Moreover, the percent of baseline luciferase signal was higher in the 3 days post-HI transplantation group. Histological cell counting of NPCs at 7 days after transplantation revealed the 3 days post-HI transplantation group was statistically significant when compared to all other groups ($P<0.05$). Phenotypically, NSCs in the 3 days post HI-transplantation group expressed nestin at close to 93% and GFAP at 2%. A greater percentage of cells in the 6 and 24 hours post-HI transplantation groups expressed GFAP with close to 8% and 5% respectively. These two acute time points of transplantation also demonstrated a higher percentage of transplanted NPCs immuno-positive for Iba-1. Non-transplanted stroked brain homogenates from the 3 days post-HI transplantation group revealed upregulation of chemokines SDF-1 and CCL2 at 7.4 and 1216 fold upregulation respectively. The adhesion molecule VCAM-1 was upregulated by 5.4 fold. Micro-array analysis revealed high expression of integrins beta-1 and alpha-4 (together comprising the VCAM-1 ligand, VLA-4) Additionally, cells expressed CXCR4 and CXCR7 (receptors for SDF-1) and CCR4 (receptor for CCL2) but not CCR2 or CCR5. Boyden Chamber migration assay revealed NSCs migrate to both MCP-1 and SDF-1 in a dose dependent manner. Conclusion: Transplanted NPC survival is optimal when delivered 3 days after HI as demonstrated by BLI and histological findings. *In vivo*, upregulation of adhesion molecule VCAM-1 and chemokines CCL2 and SDF-1 in correspondence with in-vitro expression of their ligands VLA-4 and receptors CCR4, CXCR4, CXCR7, elucidate a possible mechanism for increased transplanted NSC migration. This assertion is supported by in-vitro migration of NPCs to potent chemotaxic factors SDF-1 and MCP-1. Additionally, transplantation at 6 hours and 24 hours after stroke revealed greater percent of transplanted NPC's phagocytosed by activated microglia, as assessed by GFP/Iba-1 double positive cell counts. These data suggest a possible mechanism for significantly higher survival, migration and engraftment of NPCs transplanted at 3 days after HI.

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SIMULATED MICROGRAVITY INCREASES CELL MIGRATION AND NEUROPROTECTIVE EFFECT AFTER BONE MARROW STROMAL CELL TRANSPLANTATION SPINAL CORD INJURY

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The 3D-clinostat, simulated microgravity machine, is a multi-directional gravity device, by controlled rotation of two axes, and makes 10-3 G average over time (patented: undifferentiated pluripotent stem cell proliferation/differentiation regulation method and system, Japanese patent, publication number P2001-197182A, date of filing June. 28, 2001, P2003-9852A, date of filing January. 14, 2003, and overseas patents, WO2004/061092 A1 PCT [U.S.A., Canada, China, and Korea], P/E [Italy, U.K., Sweden, Germany, and France], 2004). We reported simulated microgravity inhibited cell differentiation such as myoblasts, osteoblasts, and stem cells. Bone marrow stromal cells (BMSCs) represent one of the potential sources of cell therapy after central nerve disorders. In the present study, we investigated the migration property and neuroprotective effect of rat BMSCs cultured in simulated microgravity using spinal cord injury model rats. Rat BMSCs were isolated from Fisher F344 rats and harvested in 1G condition. After BMSCs were proliferated, the cells were divided into two different gravity conditions: normal 1G (group 1G) and simulated microgravity using the 3D-clinostat (group CL). After 7 days culture, BMSCs were labeled with PKH-26. Spinal cord injury was induced by weighing drop method in adult female Fisher F344 rats. Immediately after injury, rats were received BMSCs of group 1G or CL through the caudal vein. Non-treated rats were injected PBS only as a control. Functional recovery was examined by Basso-Beattie-Bresnahan scale and inclined plane score at 1 to 7, 14, and 21 days. Spinal cords were removed 21 days after injury and immunocytochemically examined. Oct-4 and CXCR4 expressions were higher cells in group CL than in group 1G before cell transplantation. Locomotor function of group CL cells received rats showed more gradual improvement than non-treated and group 1G cells received rats, and a marked statistically significant difference after 4 days. In group CL cells received rats, the cavity rate of the injured space was smaller compared to group 1G cells received rats. PKH-26 positive cells mainly existed in and around the injured space, and they were observed easier in group CL cells received rats than in group 1G. PKH-26 positive cells were expressed glial fibrillary acidic protein (GFAP) rather than neurofilament. Moreover, in group CL cells received rats, the rate of Bax, apoptosis invitation marker, positive cells were significantly lower and that of Bcl-2 and Survivin, apoptosis inhibition markers, positive cells were higher compared to group 1G cells received rats. Our findings indicate that transplanted BMSCs migrate to the injury site, and mainly differentiate into astrocytes after cell transplantation in acute phase. As a result, BMSCs cultured in simulated microgravity facilitate functional recovery from spinal cord injury by increasing cell migration and neuroprotective effect.

Poster Board Number: 2046

OPTIMIZED NEURONAL CULTURE MEDIA FOR ELECTROPHYSIOLOGY APPLICATIONS

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Primary neural cells offer an indispensible tool for dissecting basic cell biology with prospective applications in cell therapy and drug discovery. Utilization of their potential largely relies on optimal culture conditions that preserves it functional properties. In particular, electrophysiological properties of neurons are known to diminish under suboptimal culture conditions. It is through electrical activity that neurons communicate with each other as well as with muscle and other end organs. Electrophysiology is the fundamental technique to assess such electrical activities to study the functions and dysfunctions in cultured neurons. Neurons are usually cultured in serum-free systems which include basal medium such as Neurobasal® supplemented with B-27® and GlutaMAX-I®. Neurons cultured in such media show lower electrophysiological spike rates relative to media supplemented with serum. We have developed an optimized medium Neurobasal Electro® and a supplement B-27 Electro® which promote higher spike rates by a mechanism involving greater sysnoptogenesis that is reflected by increased immunocytochemical marker expression of pre-synaptic Synaptophysin and post synaptic PSD-95. The immunoreactive GABA and NMDA puncta also increased 2.8- fold and 1.6-fold respectively ($n=3$, $p<0.05$) over the period of three week. Primary rat neurons cultured in Neurobasal Electro® and B-27® Electro produced higher spike rates on multielectrode arrays. The rate of cell survival was indistinguishable at day 4 between Neurobasal®/B-27® and Neurobasal Electro®/B-27® Electro assessed by Live/Dead® cell viability assay ($n=3$, 98.0 ± 0.8 vs. 97.2 ± 1.6). These results show that this media system is an improvement to Neurobasal®/B-27® for cultured networks with an increased density of synapses and transmitter receptors. Neurobasal Electro® and B-27® Electro will render better electrophysiological studies, besides normal functional studies enabling improved screening applications.

Poster Board Number: 2048

DEVELOPING AN IPSC-DERIVED NEURONAL MODEL OF AUTISM BASED ON NEUROLIGIN SIGNALING

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We have previously described a protocol to direct the differentiation of human induced pluripotent stem cells (iPSCs) into forebrain neurons by exposing them to developmentally relevant signals. Human iPSC-derived neurons (or "iPS-neurons") mature into electrophysiologically functional neurons and display a range of characteristics typical of forebrain neurons. Their maturation is facilitated by co-culturing with postnatal rat glia. Human iPS-neurons may be applied in studies investigating molecular mechanisms of human neurodevelopmental diseases such as autism. For example, we can use them in an artificial synapse formation assay where the cultured iPS-derived neurons are co-cultured with HEK293T cells that have been transfected with synapse-inducing molecules such as the Neuroligins. Human iPS-neurons are able to efficiently form Neuroligin-induced synapses onto HEK293T cells. The synapse-inducing ability autism-linked mutants of Neuroligin4 (NLGN4) can be directly compared in the same assay. NLGN4 overexpression in iPS-neurons resulted in enhanced density of presynaptic structures in three-week-old neurons. We are in the process of testing whether this enhancement correlates to an increase in synaptic activity of these neurons. These experiments provide further evidence that human iPS-neurons are a robust experimental system to investigate the effects of human disease-related mutations in neuronal synapse formation and function.

Poster Board Number: 2050

GENERATION OF IPS CELLS IN SPINAL BULBAR MUSCULAR ATROPHY (KENNEDY'S DISEASE)

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Our understanding of the pathogenic mechanisms of motor neuron disease has been limited by the absence of model systems that can adequately recapitulate disease features as they occur *in vivo*. Spinal bulbar muscular

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atrophy (SBMA), also known as Kennedy's disease, is caused by a CAG repeat expansion in the androgen receptor gene on the X chromosome, which results in a androgen-dependent toxic gain of function in the mutant protein. The length of the CAG repeat correlates inversely with the age of disease onset, with longer repeats associated with earlier onset. The iPS cell system provides a unique opportunity in which stem cells can be generated from adult patients with motor neuron disease, and then differentiated into disease-relevant progeny such as neurons and glia. We have generated iPS cells from patients with spinal bulbar muscular atrophy (SBMA) in order to generate neurons and other cells that can then be manipulated and studied *in vivo* to better understand the disease mechanism. The differentiated SBMA iPS cells will be cultured with and without the presence of dihydrotestosterone (DHT) to determine if a phenotype is affected by ligand activation. The identification of a phenotype with the iPS cell system will allow us to develop assays to test candidate therapeutics and screen for new compounds with therapeutic potential.

Poster Board Number: 2052

COMPARISON OF ADULT CANINE SKIN-DERIVED AND BRAIN-DERIVED NEUROPRECURSORS *IN VITRO*

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BACKGROUND: Neuroprecursors have been isolated from the brains of several mammalian species, including the domestic dog (Canis familiaris). Recent work has indicated neuroprecursors can also be successfully isolated from adult canine skin. Skin and the major cell types of the brain all arise from the embryonic ectoderm. Apart from this shared lineage, the similarities between skin-derived and brain-derived neuroprecursors remain unclear. AIM: To compare the proliferation capacity and differentiation potential of skin-derived and brain-derived neuroprecursors under *in vitro* culture conditions. METHODS: Periventricular regions were dissected from fresh post mortem canine brain within 4 hours of death. Following enzymatic dissociation, cells were transferred to neurosphere and then to adherent culture for analysis (NeuroCult Media, Stem Cell Technologies). Fresh excess canine skin tissue was obtained following routine veterinary surgery. Skin derived precursors were isolated and propagated according to our protocol adapted from Toma et al. Proliferative potential was quantified using the 5-ethynyl-2'-deoxyuridine (EDU, Invitrogen) assay. Spontaneous differentiation towards neuronal lineages was induced by removal of the mitogens from culture medium and by the addition of 10ng /mL of Brain Derived Neurotrophic Factor (BDNF). Gene and protein expression for various neuronal markers were carried out using PCR and immunocytochemical staining respectively. RESULTS: Under neurosphere and adherent culture conditions, both canine skin and brain-derived neuroprecursors are morphologically similar. Immunocytochemical and PCR analysis of proliferating cultures revealed expression of neural stem cell markers Nestin, NCAM and CD133. EDU proliferation assays revealed comparable proliferation rates of skin and brain-derived neuroprecursors until the second passage. Significant decreases ($p<0.05$) in the proliferation rates of skin-derived (32.63%) compared to brain-derived (74.64%) neuroprecursors were observed after the third passage. Immunocytochemical staining of spontaneously differentiated cultures from both tissue sources indicated expression of more mature neuronal markers βIII tubulin, MAP2 and NSE. However, a higher density of glial marker GFAP positive cells in brain-derived cultures (>90%) than skin-derived (<1%) was observed. Additionally, expression of GABAergic marker GAD67 was limited to skin-derived samples. These immunocytochemical findings were corroborated by PCR analysis. CONCLUSIONS: Similarities between canine skin and brain-derived neuroprecursors were found in terms of morphology and neural stem cell-like markers based on gene and protein expression. However, skin-derived neuroprecursors exhibited significantly decreased proliferation rates following third passage, whilst spontaneous differentiation suggest a preferential committal to the GABAergic lineage and decreased capacity for glial differentiation. Further research is in progress to better understand both molecular and functional properties of adult skin-derived and brain-derived canine neuroprecursors.

Poster Board Number: 2054

MODELING PRIMATE NEURAL DEVELOPMENT USING INDUCED PLURIPOTENT STEM CELLS (IPSC)

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New genomic tools provide us with high-resolution information about the alterations that may have resulted in the evolution of our own species. However, all information available to date for comparative studies between humans and our closest relatives come from DNA/RNA samples extracted from preserved (post-mortem) tissues. These samples don't always fairly represent the distinctive traits of live cell behavior. The identification of differences in genetic makeup between related species needs to be translated into phenotypic divergence. It is likely that some of these alterations will be subtle, impacting specific cell types at precise time points during development. Pluripotent stem cells can differentiate into several cell types of the body, recapitulating early stages of an organism's development. In this study, we characterize induced pluripotent stem cells (iPSC) derived from somatic cells of humans, bonobos and chimpanzee's individuals. Bonobo's and chimpanzee's iPSC behave similarly to human cells regarding self-renew capacity and pluripotency potential. Using a neural differentiation protocol we show that all species were able to generate electrophysiologically active neurons. Moreover, we demonstrate that human cells can recapitulate differences from bonobo and chimpanzee such as specific transcriptional and biochemical modifications, caused by genomic mutations that occurred after the split of the human lineage. This approach provides a unique biological resource to elucidate the phenotypic differences between human and apes at specific time points during cellular speciation. Our culture model could bring new insights to human adaptation, with potential consequences to biomedical research and basic biology of the species.

Poster Board Number: 2056

MODULATION OF SPREADING AND MIGRATION OF ASTROCYTES GENERATED FROM NEURAL STEM/PROGENITOR CELLS ON ELASTIC HYDROGELS

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Background: The mechanical properties of cell-scaffold materials affect some cell behavior, the differentiation pathway of somatic stem cells and the motility of fibroblasts. The scaffold designing taken account of their properties are essential for controlled behavior of stem cells in tissue engineering applications. We investigated the cell motility and spreading of the differentiated cells generated from mouse cerebral neural stem/progenitor cells (NSPC) formed neurosphere on type I collagen gels and polyacrylamide gels with storage elastic modulus (G') from 100-2000 Pa. Methods: The stiffness of collagen gels were adjusted by carbodiimide cross-linking technique. Polyacrylamide gels were prepared with acrylamide/bis-acrylamide mixture,

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containing 10% acrylamide and bis concentrations ranging from 0.26 to 0.03%. After gelation, type I collagen molecules were bound on the surface of polyacrylamide gels by photoactivated crosslinker. NSPC which formed small neurosphere by serum-free culture for three days, were induced differentiation in the medium containing 1% fetal bovine serum on respective collagen gels and polyacrylamide gels. Cell differentiation was evaluated by immunofluorescence staining with anti-tubulinβIII (neuronal marker) and anti-GFAP antibodies (astrocytic marker). The cell motility and spreading were by image analysis based on cell area and boundary length. Real-time RT-PCR analysis was performed on differentiated cells to evaluate changes in Rho family gene (Cdc42, Rac1, RhoA) expression. Results: In every gel conditions, the ratio of neurons were 5-10%, astrocytes were 90-95%. We could not observe the significant effect on cell differentiation lineage. However, spread area of astrocytes generated from neurosphere-formed NSPC was more extensive on harder gels (G′ > 1000 Pa) in both hydrogels. The expression level of cdc42 and Rac1 also were higher on harder gels. Neurons generated from neurosphere-formed NSPC migrated on all hydrogels. Conclusion: The stiffness of hydrogels (G′ > 1000 Pa) is important for the migration and spreading of astrocytes from neurosphere.

Poster Board Number: 2058

DRONC CASPASE EXERTS A NON-APOPTOTIC FUNCTION TO SUPPRESS PHOSPHO NUMB-INDUCED EXCESS NEURAL STEM CELLS IN *DROSOPHILA*

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Drosophila neural stem cells, or called neuroblasts, have been instrumental for investigating mechanisms by which an appropriate balance of stem cell self-renewal versus differentiation is accomplished. *Drosophila* Numb protein controls this process through its predominant inheritance by the differentiating daughter cell. How Numb restricts the proliferation and self-renewal potentials of the recipient cell remains enigmatic. Here we show that phosphorylation at conserved sites regulates the tumor suppressor activity of Numb. Enforced expression of a phospho-mimetic form of Numb (Numb-TS4D), or genetic manipulation boosting phospho-Numb level, attenuates endogenous Numb activity and causes ectopic neuroblast formation. This effect on neural stem cell (NSC) homeostasis occurs only in type II neuroblast lineage. The prominent brain tumor-like phenotype induced by Numb-TS4D provides an excellent system to identify novel molecules controlling NSC homeostasis. We identify that Dronc caspase is a novel binding partner of Numb, and demonstrate that overexpression of Dronc suppresses Numb-TS4D effects in a non-apoptotic, and possibly non-catalytic manner. Conversely, depletion of Dronc activity enhances the over-proliferation phenotype upon impairment of Numb function. This result indicates that normal Numb-Dronc interaction is very important for NSC homeostasis. Our findings reveal a molecular mechanism regulating Numb activity and implicate a novel role for Dronc caspase in regulating NSC homeostasis. Dr. Ouyang conducted his work when he was at Stanford University.

Poster Board Number: 2060

A ROLE FOR THE SNAIL TRANSCRIPTION FACTOR IN THE SURVIVAL AND MAINTENANCE OF DEVELOPING RADIAL PRECURSOR CELLS

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The transcriptional repressor Snail has been implicated in the development of stem cells in both *Drosophila* and *C. elegans*, regulating survival in *C. elegans*, and self-renewal versus differentiation in *Drosophila*. In mammals, Snail has been shown to regulate the epethelial to mesenchymal transition (EMT) during development and in cancer, a process that has been implicated in regulating stem cell states. We have therefore asked whether Snail might play a role in regulating the biology of neural stem cells, focusing upon the developing cortex. We show that Snail is expressed in the embryonic cortex, where it is enriched in radial precursors and in newly-born cortical neurons. Acute genetic knockdown of Snail in developing cortical precursors in vivo results in cellular apoptosis, specifically within Pax6-positive radial precursors. This apoptotic phenotype can be rescued by coincident knockdown of p53, indicating that Snail normally supports precursor survival by inhibiting a p53-dependent apoptotic pathway. However, while p53 knockdown rescues the apoptosis, it does not fully rescue the Snail knockdown phenotype suggesting that Snail plays other roles in radial precursors. In support of this idea, overexpression of Snail within cortical precursor cells in vivo resulted in an increase in the proportion of Pax6 radial precursors, suggesting that Snail also regulates self-renewal in a p53-independent fashion.

Poster Board Number: 2062

A COMBINATORIAL APPROACH FOR DEVELOPMENT OF SYNTHETIC SURFACES FOR STEM CELL CULTURE

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Development of scalable, reproducible, and regulatory-friendly synthetic surfaces for stem cell culture has been a challenge for cell therapy applications. Combinatorial chemistry and parallel screening approaches have been used to identify biological and biomimetic surfaces to culture stem cells with varying success. Here, we report a combinatorial approach that enabled the identification and validation of an optimized synthetic surface for long term culture of neuronal progenitor stem cells in chemically defined media. A variety of acrylate based formulations were polymerized in 96-well microplates. Adhesion peptides from extra cellular matrix proteins were conjugated to the acrylate surfaces through carboxyl groups to provide biospecific binding epitopes for the library. ReNcell VM human neural progenitor cells (Millipore) were propagated on the library using serum free medium. Cell proliferation was evaluated either by luminescence ATP (Promega) or CyQUANT (Life Technologies) assays. Neurons in the differentiated cultures were identified using β-Tubulin-III immunochemical assay (Millipore). Passage studies were done in coated and gamma sterilized 6 well plates. In the screen "hits" were identified based on proliferation data. Of these, the P75 surface enabled differentiation into neurons similar to the Laminin protein standard. Multi-passage study was done to further validate the surface. P75 performance was comparable to Laminin for up to 9 passages based on doubling time, and fold of expansion. Cells retained normal karyotype, expression of phenotypic markers, and ability to differentiate into neurons after long-term passaging. We have developed an efficient process for generating combinatorial libraries of biomimetic materials in microplate formats.

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The library can be tuned to provide a broad range of chemical structure, mechanical and surface properties. It can also be functionalized with a range of peptide epitopes or other molecules at varying densities to further modulate cell specific attributes of adhesion, release etc. Here, we used this strategy to identify the most optimal synthetic surface for the culture of ReNcell. Laminin is a well established culture surface for ReNcells and other neuronal cell lines. Thus, we expected Laminin-based adhesion epitopes to be successful in this screening against neuronal cells. However, none of the tested laminin peptide containing surfaces was found to be a hit. Interestingly, all our "hits" contained RGD epitopes; although not all RGD epitopes were equivalent in culture performance. In addition, the identified hit P75 is identical to the surface that was previously reported by us for long term culture of human embryonic stem cells and recently commercialized as Corning® Synthemax™ Surface. In conclusion, our combinatorial approach has been very powerful in successfully identifying optimal synthetic surfaces for various cell culture applications.

CANCER CELLS

Poster Board Number: 2066

NK-92 INFUSIONS IMPROVE SURVIVAL IN NOD/SCID GAMMA NULL MICE BEARING ACUTE MYELOID LEUKEMIA XENOGRAFTS

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Introduction: Survival with acute myeloid leukemia (AML) remains poor particularly for high risk patients who lack a matched stem cell donor. NK-92 is a human permanent natural killer (NK) cell line in a phase I clinical trial for relapsed and refractory hematologic malignancies. Irradiation of NK-92 prevents proliferation and tumour formation in patients. We recently showed that NK-92 targets leukemic stem cells (LSCs) in the cell line KG1 and in five primary AML samples using a clonogenic assay. Here, we establish a leukemia xenograft model in NOD/SCID gamma null mice to evaluate the therapeutic efficacy of NK-92 and the impact of irradiation of NK-92 on *in vivo* cytotoxicity. Methods: 3.2x106 primary AML cells were injected via tail vein into NOD/SCID gamma null mice irradiated with 325 cGy and maintained on Baytril. 10-20x106 NK-92 (+/- irradiation with 1000 cGy) were infused via tail vein every 5-7 days to a total of 30x106 or 60x106 cells in two separate experiments. Mice were monitored for leukemia and sacrificed at humane endpoints. Autopsies were performed on select mice from each cohort, including flow cytometry of bone marrow (BM) and splenocytes to determine leukemic burden. Flow cytometry staining panel was designed to detect primary AML cells (class I HLA A, B, C) including the leukemic stem cell fraction (CD34, CD38) and NK-92 (CD56). Kaplan Meier survival curves were generated to compare survival in control and treatment groups. Results: In the pilot, NSG mice infused with AML developed signs of leukemia at day 50 including weight loss and decreased activity and died or required sacrifice by day 60. Autopsy revealed enlarged spleens and pale fragile bones relative to controls. Treatment of AML-infused NSG mice receiving 10x106 non-irradiated NK-92 weekly x3 increased median survival from 60 to 85 days (log rank test $p<0.01$). One mouse of 10 survived long-term to 7 months age and on sacrifice had no evidence of splenomegaly or detectable leukemia in BM or splenocytes by flow cytometry. Two mice infused with non-irradiated NK-92 weekly developed signs and symptoms of leukemia at day 90 requiring sacrifice with evidence of NK-92 in the spleen and BM. In a follow-up experiment, total dose was doubled to 60x106 with intent to use 20x106 cells per infusion. Three of 25 mice infused with 20x106 NK-92 had transient acute reactions and minimal activity with apparent recovery after 10 minutes. Further doses of NK-92 were reduced to 10x106 to complete the total 60x106 dose. All mice with AML developed weight loss and decreased activity requiring sacrifice between day 50-60 as with the pilot study. At day 65 survival assessment revealed: non-irradiated NK-92 only (3/5), irradiated NK-92 only (3/5), AML only (0/5), AML + irradiated NK-92 (2/10), AML + non-irradiated NK-92 (1/5). Mice with AML infusions developed splenomegaly and had near 100% BM engraftment of human leukemia cells. Summary and conclusion: We show that NK-92 enhances survival in mice bearing primary human AML and is potentially curative. Individual doses of 10x106 are tolerated well by tail vein, but 20x106 leads to acute stress and possibly early mortality, most likely due to the high cell burden. There is no difference in survival between non-irradiated and irradiated NK-92 in the ongoing experiment and is of relevance because only irradiated cells are currently used in patients. We also report for the first time engraftment of NK-92 in an animal model indicating the high degree of permissiveness of NSG mice irradiated with 325 cGy.

Poster Board Number: 2068

NOTCH SIGNALING IS UPREGULATED IN THE CHRONIC PHASE OF CHRONIC MYELOID LEUKEMIA

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Notch signalling is critical for haemopoietic stem cell self-renewal and survival. Chronic Myeloid Leukaemia (CML) is a stem cell disease characterised by the presence of the Philadelphia (Ph) chromosome, and subsequent expression of the BCR-ABL oncogene. The well established role for Notch signalling in human T-cell acute lymphoblastic leukaemia (T-ALL) and the reported interaction between Notch and ABL in different developmental contexts in *Drosophila* raise the possibility that Notch signalling may be dysregulated in CML. Therefore, the aim of the study was to investigate whether Notch signalling is altered in CML and to study possible crosstalk between Notch signalling pathway and BCR-ABL in CML. The gene expression patterns of all four human Notch genes and the Notch target gene HES1 were studied in CD34+ stem and progenitor cells isolated from CML patients. Poly-A PCR followed by real time PCR analysis was used to quantitate gene expression levels in comparison with levels in equivalent populations isolated from normal bone marrow (NBM). The expression of Notch1 receptor protein levels expressed on the cell surface was also investigated by flow cytometry. Results showed an up-regulation of Notch1 and Notch2 genes and the target gene Hes1 on the most primitive CD34+ Thy+ subset of CML CD34+ cells as compared with NBM. In addition, Notch1 receptor protein was expressed in distinct lymphoid and myeloid progenitors within the CD34+ population of CML cells. These results suggest that Notch signalling may be highly activated in CML primitive progenitors. To investigate the possible crosstalk between Notch and ABL *in vitro* human cell line model systems were assessed as possible models to study the interactions between Notch and ABL signalling and the FACS based P-crkl assay was optimised as a rapid method to assess ABL activity. The data showed that K562 and ALL-SIL cell lines are sufficient model systems to investigate the cross-talk between the Notch and ABL signalling pathways. The imatinib induced inhibition of ABL activity in K562 and ALL-SIL cells resulted in significant up-regulation of Notch activity as assessed by Hes1 expression. Similarly, GSI inhibition of Notch signalling in K562 cells resulted in hyperactivation of ABL kinase activity as assessed by P-crkl levels. The antagonistic relationship between Notch and ABL signalling observed in cell lines were further confirmed in CD34+ cells from chronic CML patients. Treatment of CD34+ CML cells with imatinib led to significant up-regulation of Notch activity whereas inhibition of Notch signalling with GSI in CD34+ CML cells resulted in increased ABL activity. It can be concluded therefore, that Notch signalling may be dysregulated in the chronic phase of CML. In addition, the data presented in this project demonstrate for the first time the cross-talk between Notch signalling and ABL signalling in cell line model systems as well as in primary CD34+ CML cells. Future work is required to address the possible mechanisms that under-

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lie the findings observed here and to investigate the biological consequences of the interplay between Notch and ABL signalling in CML.

Poster Board Number: 2070

EFFECTS OF RESVERATROL ON HL-60 LEUKEMIA AND RAJI LYMPHOMA CELLS: A MOLECULAR AND IMMUNOHISTOCHEMICAL STUDY

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Introduction: Resveratrol is a polifenolic compound found in grapes, red wine, peanuts and huckleberries. In previous studies Resveratrol's antioxidant, anticancer, cell protective and proapoptototic characteristics were shown. The aim of our study was to demonstrate the microRNA profile and to determine the apoptosis ratio by immunohistochemical methods in promyelocytic leucemia HL-60 and lymphoma Raji cell lines after resveratrol administration. Method: Routine cell culture technics for HL60 and Raji cells were done: cultured, passaged, treated Resveratrol and counted vital cells. Then, total was RNA isolated and continued with microarray for microRNA profile and with IHC-Apoptag (TUNEL) for apoptosis ratio. Results: We determined the increases and decreases of microRNA's after resveratrol administration. Besides we found a twofold higher apoptosis ratio in resveratrol administrated HL-60 group compared to control group. No statistical significance was found between control raji and resveratrol administrated raji groups but a tendency of increasing was found in resveratrol administrated group. Conclusion: Further microRNA analysis is needed in leukemia and lymphoma because of its therapeutic potential.

Poster Board Number: 2072

TRANSCRIPTION FACTOR BCL11A DUALITY IN NORMAL AND LEUKEMIC STEM CELL FORMATION

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The biological and genetic differences of normal haematopoietic stem cells (HSC) and leukaemia stem cells (LSC) are not well known. Likewise how a normal HSC converts to LSC is poorly understood. We aimed to find essential differences between HSC and LSC by studying the roles of B-cell leukaemia/lymphoma 11a (Bcl11a) a tumour suppressor with dual function during normal and abnormal haematopoiesis. Bcl11a/Evi9/CTIP1 is a transcription factor that is activated in patients with a rare translocation involving the IG locus and is found as a retroviral insertion in the BXH2 mice leukaemia. We demonstrated that deletion of Bcl11a in mice is lethal, it blocks early B cell development, causes abnormal T cell development, does not affect myeloid or erythroid development, and results in leukaemia when transplanted into lethally irradiated mice. Therefore, to identify similarities or differences between HSC and LSC during self-renewal, survival, differentiation, and proliferation we derived Bcl11a-/- LSC lines from mice transplanted with Bcl11a-/- foetal liver cells and by co-cultures with OP9 stromal cells. These LSC lines showed high expression of stem cell surface markers (c-Kit and Sca-1), T cell markers (CD4 and CD8), B cell markers (B220 and CD43), very low expression of myeloid (Gr1 and Mac-1) and erythroid markers (TER119E and CD71) by flow cytometry. Additionally, the Bcl11a-/- LSC showed Side Population by Hoechst and stem cell renewal ability by single cell assays, which are both characteristics of HSC. They also gave rise to myeloid and erythroid colonies when assayed by colony formation assays (CFU-C). To establish the gene expression differences between HSC and Bcl11a-/--LSC we performed microarray assays and we began to compile differentially expressed genes; we also performed comparative genome hybridization, and FISH to determine chromosome integrity. To determine the stem cell ability *in vivo* we transplanted very low cell numbers (50 cells) of double and single positive Sca-1+ and c-Kit+ sorted cells. Our preliminary results indicate that as little as 50-sorted Sca1+/c-Kit+ cells cause leukaemia at 6 months post transplantation. In addition, we determined that Notch inhibitors and rapamycin could inhibit the growth of Bcl11a-/--LSC. We believe that our studies will identify key differences and similarities between HSC and LSC, which are now lacking. These key findings will shed light to understand how normal HSC convert to LSC, which is needed to develop therapies that target distinctively the LSC from the HSC.

Poster Board Number: 2074

IDENTIFICATION OF T-LYMPHOCYTIC LEUKEMIA-INITIATING STEM CELLS RESIDING IN PATIENTS WITH ACUTE MYELOID LEUKEMIC DISEASE

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Xenotransplantation of acute myeloid leukemia (AML) into immunodeficient mice has been critical for understanding leukemogenesis *in vivo* and defining self-renewing leukemia-initiating cell subfractions (LICs). Although AML-engraftment capacity is considered an inherent property of LICs, substrains of NOD/SCID mice that possess additional deletions such as the IL2Rγcnull (NSG) have been described as a more sensitive recipient to assay human LIC function. Using 21 AML-patient samples, 43% demonstrated no detectable engraftment in NS and were categorized as AMLs devoid of LICs. However, 33% of AML patients lacking AML-LICs were capable of engrafting NSG recipients, but produced a monoclonal T-cell proliferative disorder similar to T-ALL. These grafts demonstrated self-renewal capacity as measured by *in vivo* serial passage and were restricted to CD34-positive fraction, and were defined as LICs. Molecular analysis for translocations in MLL genes indicated that these AML patient-derived LICs all expressed the MLL-AFX1 fusion product. Our results reveal that the *in vivo* human vs. xenograft host microenvironment dictates the developmental capacity of human LICs residing in patients diagnosed with AML harboring MLL mutations. These findings have implications both for the basic biology of CSC function, and for the use of *in vivo* models of the leukemogenic process in preclinical or diagnostic studies.

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Poster Board Number: 2076

BMP2 SENSITIZES INTRATUMORAL GLIOBLASTOMA HUMAN STEM CELLS TO TEMOZOLOMIDE BY AFFECTING HIF-1α STABILITY

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Glioblastoma multiforme (GBM) is the most common malignant tumour occurring in the central nervous system (CNS), actually treated by surgical removal followed by radio- and Temozolomide (TMZ)-based chemotherapy. We recently showed that GBM tumor mass, concentrically sampled through image guided surgery, is characterized by a central and partially necrotic core, with a high HIF-1α expression, that positively correlate with tumor aggressiveness and cancer stem cell maintenance, and enriched in immature CD133+, Nestin+ and MGMT+ cells that result to be resistant to TMZ *in vitro* treatment. We and others recently reported on the role of BMPs in promoting astro-glial differentiation and in reducing cell growth in GBM-derived cells, considering BMPs treatment a promising therapeutic approach for brain cancer. Since we demonstrated that more differentiated cells in the GBM mass expressed low levels of MGMT and were hence sensitive to TMZ treatment, our aim was to investigate whether a pro-differentiating treatment, based on BMP2 administration, would increase resistant hypoxic primary GBM derived cells sensitivity to TMZ. Here we show that TMZ alone was able to reduce the number of CD133+ and Nestin+ cells by promoting GBM cell differentiation, as shown by the increase in CD24+ and β-III-tubulin+ cells, majorly in the peripheral areas of the tumour and confirmed that more undifferentiated cells (i.e. cancer stem cells) were resistant to TMZ treatment. Confirming our hypothesis, BMP2 was able to sensitize resistant hypoxic GBM cells to TMZ treatment since BMP2 and TMZ combination therapy induced strong apoptosis and differentiation *in vitro* compared to BMP2 or TMZ alone. As down-regulation of HIF-1α occurred following BMP2/TMZ treatment and considering the previously described correlation existing between hypoxia and MGMT expression, we tested whether MGMT expression was dependent on HIF-1α expression and found that down-modulation of HIF-1α signalling was able to partially ablate MGMT expression, thus providing a possible sensitization mechanism of GBM cells given by BMP2 administration. In conclusion in this work we show that BMP2 is able to induce a strong differentiation program in GBM derived stem cells, inhibit HIF-1α signalling and suppress MGMT expression, thus sensitizing more resistant GBM derived cancer stem cells to TMZ treatment.

Poster Board Number: 2078

ONCOGENIC VARIANT EGFRVIII DEFINES A CANCER STEM CELL POPULATION IN GLIOBLASTOMA MULTIFORME

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Glioblastoma multiforme (GBM) is the most common and deadly primary brain tumor. Median survival is only 14 months, with tumor recurrence in >90% of cases. Research suggests cancer stem cell (CSC) or tumor initiating populations exist in GBM that mediate tumor initiation, local invasion, and recurrence. Putative CSCs have been identified via cell surface markers CD133 and CD15, but targeting is complicated by their expression in normal non-cancerous tissue. Here we report evidence that a naturally occurring tumor-specific variant of the epidermal growth factor receptor (EGFR), called EGFRvIII, is a novel CSC marker in GBM. EGFRvIII is an exon2-7 deletion mutant lacking part of the extracellular ligand binding domain, rendering it ligand independent and constitutively active. Found in 15-60% of GBM, EGFRvIII can be the result of either a genomic rearrangement or alternative splicing, and the rearrangement is often associated with amplification of the EGFR locus. We found EGFRvIII expressed in 40% of GBM by immunohistochemistry (IHC). Expression was highly correlated with EGFR locus amplification, assessed by fluorescence *in situ* hybridization (FISH), and the EGFRvIII genomic rearrangement, determined by long-range PCR. Interestingly, EGFRvIII expression is sporadic and restricted to sub-regions within the tumor, often displaying perivascular clustering. To better understand the contribution of this minority population to tumorigenesis and CSC phenotypes, we performed flow cytometry analysis and sorting. Flow cytometry revealed a higher percentage of EGFRvIII positive tumors (71%), with 1-25% of the cells being positive in each case. Because EGFRvIII expression can be sporadic, IHC may present a sampling bias that has lead to an overall underestimation of the prevalence of EGFRvIII. To understand the functional relevance of EGFRvIII, we sought to devise a method of maintaining EGFRvIII expression *in vitro*. EGFRvIII gene and protein expression is lost when cells are grown under standard serum-culture techniques, but can be maintained when primary GBM are cultured as neurospheres. Importantly, EGFRvIII expression in spheres can be maintained for greater than 10 passages, and EGFRvIII is lost when spheres are exposed to differentiating conditions. Furthermore, EGFRvIII is frequently expressed in the putative CSC CD133+ population of GBM. Flow cytometry demonstrates EGFRvIII expression in 81% (13/16) of primary GBMs expressing CD133, and there is a significant enrichment for cells expressing both proteins. Notably, cells that express both EGFRvIII and CD133 display greater self-renewal, as shown through limiting dilution sphere forming assays, and increased tumorigenicity when compared to either the single positive or double negative populations. Tumor formation was observed in 80% (15/18) of mice injected intracranially with 1000 EGFRvIII+/CD133+ cells, as compared to 25% (5/18) and 33% (4/12) of mice injected with EGFRvIII-CD133+ or EGFRvIII+/CD133- populations, and as many as 100,000 EGFRvIII-CD133- cells could not form tumors. Finally, we created a bispecific antibody that targets cells expressing both EGFRvIII and CD133 and found that antibody-mediated cell killing of this population leads to a significant decrease in the sphere and tumor forming capacity of GBM sphere lines. Together our data suggests that EGFRvIII is expressed in the CSC population of GBM, and targeting of this receptor represents an excellent therapeutic strategy.

Poster Board Number: 2080

MIR-338-3P MEDIATES NOTCH-REGULATED SELF-RENEWAL IN GLIOBLASTOMA STEM CELLS

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Glioblastoma (GBM) is the most common malignant brain tumor in human with extremely poor prognosis and new treatment for this deadly disease is desperately needed. Cancer stem cells (CSCs) have been prospectively isolated from many types of cancer, including GBM. Finding genes or signal pathways that regulate GBM CSCs may help develop novel therapeutic strategy. We have demonstrated recently that Notch pathway blockade by gamma-secretase inhibitor (GSI) depletes GBM CSCs and prevents tumor propagation both *in vitro* and *in vivo*. In the current study, we found that miR-338 was down-regulated by GSI treatment in GBM CSCs. When we introduced miR-338-3p into GBM neurospheres by lentivirus, we found that miR338 decreases clonogenesis of GBM neurospheres and nestin-positive

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population, indicating that CSC population was reduced. Furthermore, we found miR-338 reduces GBM neurosphere growth through decreased proliferation and induced apoptosis. In addition, we found that over-expression of miR-338-3p is associated with better survival in GBM patients. Interestingly, miR-338-3p was expressed at a lower level in the core of tumor whereas at a higher level in the peripheral region. miR-338-3p also prevents GBM neurosphere migration by Boden chamber assay. Furthermore, we found that miR-338-3p reduces HDAC expression at protein level. Finally, we found that miR-338-3p has no growth-inhibition effect on normal human neural stem cells, suggesting that miR-338-3p may spare normal stem cells when it was used to target GBM CSCs. Taken together, our data suggest that miR-338-3p medicates Notch-regulated GBM CSC self-renewal and migration through HDAC protein, indicating that miR-338-3p can be used to target CSCs in GBM.

Poster Board Number: 2082

HYPOXIA AND WNT PATHWAY CO-OPERATION PROMOTES PRIMARY GLIOBLASTOMA DERIVED STEM CELLS REPROGRAMMING TOWARD NEURONAL FATE

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The definition of tumour niche signals involved in brain tumours stem cell maintenance is crucial to understand the processes of glioblastoma onset, progression and relapse. In this study we treated primary glioblastoma (GBM) derived cells, constantly maintained in a hypoxic microenvironment, with exogenous Wnt3a molecules and we evaluated the phenotypic reprogramming effects of Wnt pathway activation in the undifferentiated GBM cells both in our hypoxic *in vitro* conditions and in cells acutely exposed to environmental 20% O2. We found that exogenous Wnt3a promotes phenotypic reprogramming of GBM derived cells toward a neuronal fate. Indeed, we found an increase in β-III tubulin positive cells after Wnt3a treatment with a parallel decrease of Nestin and GFAP positive cells. Moreover, we found that hypoxia enhances the activation of the pro-neurogenic Wnt pathway target genes and that Wnt3a promotes neuronal differentiation majorly in GBM stem cells (CD133+) under hypoxic conditions. The mechanism that regulates GBM stem cells differentiation involves a Wnt-mediated suppression of Notch pathway activity. Moreover, Notch1 suppression under hypoxia significantly contributes to GBM stem cells neuronal differentiation and cell cycle block. In this study we point to Wnt signalling activation as a new potential molecular strategy to be used, possibly in combination therapy, in order to promote GBM differentiation, tumour cell growth inhibition and eventually cancer cell death.

Poster Board Number: 2084

DECREASE OF NEUTROPHIL INFILTRATIONS AND NECROSIS FOLLOWING SUBCUTANEOUS XENOGRAFTING OF CANCER STEM-LIKE CELLS DERIVED BY SPHEROID CULTURE FROM U87MG GLIOBLASTOMA CELL LINE

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Recent neuro-oncological studies revealed that a small subset of cells derived from glioblastoma(GBM), has specific characteristics of cancer stem-like cells such as cancer re-initiation and chemo-resistance. Several literatures showed that GBM cells were capable of forming tumor neurospheres *in vitro* in serum-free neuronal stem cell media. Compared to GBM cells cultured in serum-contained media, cancer cells not only expressed stemness markers, such as CD133, site-specific embryogenic antigen-1 (SSEA-1), or Nanog within neurospheres, but also possessed higher tumor re-initiation capabilities as demonstrated by nude mice xenotransplantation. The detailed histopathological observations in the xenografts, however, haven't been fully addressed. In our study, we generated neurospheres from U87MG, which was a representative cell line of GBM, by using the methods of neurospheroid culture in serum-free media containing epidermal growth factor (EGF) and basic fibroblast growth factor (FGF). Both cells from serum-contained cultures and serum-free neurospheres were then injected into nude mice flanks for subcutaneous xenografting. The histopathological observations were processed for comparison of different origins. There were marked differences in the xenografts derived by both different culture cells. Tumors derived by the xenografting of the serum-contained cultured cells demonstrated bigger size and higher growth rate compared to the xenografting of the cells from the neurospheres. Pathologically, extensive necrosis and neutrophil infiltrations were obvious. Tumors derived by serum-free neurospheres grew slowly in size, but they were infiltrated by little number of neutrophils with nearly absence of necrosis. In both xenografts, CD133 and SSEA-1 were positively stained in neutrophils but not among tumor cells. In summary, our results indicate the histopathological differences between the subcutaneous tumors derived from cancer cells and cancer stem-like cells of GBM neurospheres. The tumors of the cell xenograftings from neurospheres show dramatically more tolerable to hypoxia, which in turn resulted in less inflammatory cell infiltration and necrosis. In addition, the utility of CD133 and SSEA-1 as surface markers for deriving cancer stem cells by sorting from tumors should be carefully identified since neutrophils also express both markers.

Poster Board Number: 2086

TGFβ REGULATES HUMAN BREAST TUMOUR INITIATING CELL NUMBERS VIA NEDD9-MEDIATED SMAD AND SRF COORDINATED REGULATION OF GENE EXPRESSION

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Human breast tumours contain a subset of cells responsible for tumour propagation and metastasis known as breast tumour-initiating cells (BTICs). We show here that TGFβ increases or decreases BTIC number in a cell-con-

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text dependent manner. In normal mammary epithelium TGFβ has similar contrasting effects in distinct epithelial cell subpopulations. A TGFβ synexpression signature (TβSC-A), derived from cell lines with TGFβ-induced BTIC activity, is significantly enriched in gene sets derived from stem cells. This signature is predictive of worse clinical outcome in breast cancer patients. NEDD9, a top gene in TβSC-A, mediates the TGFβ-induced BTIC activity. NEDD9, through a positive feedback loop, links TGFβ/Smads and Rho-actin-SRF signals to converge at regulatory regions to regulate TβSC-A genes and increase BTIC number. We propose that combined targeting of Rho-actin-SRF and TGFβ pathways could represent a new strategy to eliminate BTICs.

Poster Board Number: 2088

NEUROPILIN-1 IS ESSENTIAL FOR MAMMOSPHERE FORMATION BY HUMAN BREAST CANCER STEM CELLS

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Neuropilin-1 (Nrp1) is a multifunctional transmembrane protein, which acts as a coreceptor for several mediators including VEGF, class 3 semaphorins, TGF-beta, hepatocyte growth factor (HGF) and platelet-derived growth factor. TGF-beta acts on breast cancer stem cells and promotes mammosphere formation. We have recently shown that Nrp1 can capture and activate latent TGF-beta-1 (LAP-TGF-beta-1) on the membrane of cancer cells, and acts as a coreceptor for TGF-beta by augmenting canonical Smad2/3 signaling. Since Nrp1 acts as a coreceptor for several growth factors potentially relevant to cancer stem cells (CSCs), including TGF-beta, we hypothesized that it plays an important role in CSC biology. We examined Nrp1 in breast cancer cells lines by flow cytometry, and found that it was frequently co-expressed with other stem cells markers. We observed that an aryl hydrocarbon receptor agonist drug, tranilast, which inhibited breast CSCs also markedly suppressed Nrp1 expression. To examine the role of Nrp1 more specifically, we performed Nrp1 knockdown with siRNA. Knockdown severely interfered with mammosphere formation, such that there were much fewer spheres and they were small. Cell survival was moderately decreased by knockdown. Several mechanisms might account for these findings, and we are currently examining how Nrp1 alters the response of CSCs to growth factors. Because CSCs exhibit enhanced NF-kB signalling, we examined this pathway. Without knockdown of Nrp1 this pathway was indeed activated, but in cells with knockdown NF-kB signaling was not detectable above background. We conclude that Nrp1 is expressed by breast CSCs, enhances NF-kB signaling and is essential for mammosphere formation.

Poster Board Number: 2090

GENERATION AND MOLECULAR PROFILING OF BREAST EPITHELIAL CELLS WITH CANCER STEM CELL PROPERTIES

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Stem cell hypothesis of cancer predicts that most of the cancers originate in normal stem/progenitor cells. However, the molecular mechanisms that govern the tumorigenic conversion of these cells remain elusive due to lack of suitable model system. In this study, we have generated an immortalized mammosphere-derived breast epithelial cell line (NBLE) which retains stem cell properties. We observed that the CD44+/CD24- fraction of NBLE cells show deregulated Notch, Wnt and Hedgehog pathways, exhibit Epithelial-Mesenchymal Transition (EMT), over-express stemness related genes and generate adenocarcinomas in immunocompromised mice and thus resemble breast cancer stem cells. These cells harbor chromosomal aberrations such as X-chromosome deletion and gain of chromosomes 8, 20 and 22 which is commonly observed in naturally occurring breast cancers. Molecular profiling showed that the tumorigenic NBLE cells exhibit gene expression profile similar to CD44+/CD24- fraction from primary breast cancer samples. Further, microRNA profiling showed significant down-regulation of miRNAs such as miR200a/b/c, miR205, miR141 and miR421. Down regulation of these miRNAs has been previously shown to result in induction of EMT and increased levels of stem cell markers such as Bmi1. The transformed NBLE cells showed activated DNA damage response which has been shown to convert normal human mammary epithelial cells into tumorigenic cells. Thus, deregulated self-renewal pathways, induction of EMT, triggering of DNA damage response and karyotypic alterations are the likely contributors for the transformation of these mammosphere-derived NBLE cells. Thus, the NBLE cells provide a novel experimental system to understand the molecular changes associated with the transformation of mammary stem/progenitor cells and can be used for screening drugs targeted against breast cancer stem cells.

Poster Board Number: 2092

PODOCALYXIN IS A KEY REGULATOR OF BREAST CANCER PROGRESSION AND METASTASIS

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Recently, we found that podocalyxin (Podxl), a CD34-sialomucin normally expressed on hematopoietic progenitors and vascular endothelia, is upregulated on a subset of primary breast tumors associated with poor clinical outcome. To determine whether podocalyxin directly regulates breast cancer cell behavior, we ectopically expressed podcalyxin in the human breast cancer cell line, MCF7. MCF7 cells express low levels of podocalyxin and make well-polarized epithelial monolayers *in vitro*, relatively normal mammospheres in 3D culture, and, are non-metastatic. Upon ectopic expression of podocalyxin, MCF7 "bulge apically", produce apical and lateral microvilli, are less adhesive *in vitro*, and are delayed in targeting integrins to the basolateral surface. In addition, podocalyxin enhances solid tumor expansion in MCF7 subcutaneous xenografts (RAG2-/- mice). In contrast to MCF7, MDA.231 cells express high levels of endogenous podocalyxin, exhibit poorly polarized monolayer and mammosphere architecture *in vitro* and form "metastatic" lung tumors *in vivo*. Using Podxl-targeted shRNA vectors, we show that expression of podocalyxin is required for MDA.231 motility *in vitro* and *in vivo*. The impaired motility phenotype of Podxl-deficient MDA.231 cells was as robust as Cxcr4- and Ccr7-deficient MDA.231 lines. Both CXCR4 and CCR7 have key roles in the motility of breast cancer cells and we have previously found that podocalyxin enhances CXCR4-mediated chemotaxis in hematopoietic cells. Thus, up-regulation of podocalyxin may be a key event in tumor progression and likely endows cells with an enhanced ability to sense chemokines and metastasize to peripheral sites.

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Poster Board Number: 2094

INVOLVEMENT OF THE NERVE GROWTH FACTOR ON THE BALANCE BETWEEN PROLIFERATION AND QUIESCENCE IN BREAST CANCER STEM CELLS

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Despite advances in early detection of cancers, disease relapse still occurs in a not insignificant percentage (20-30 %) of patients with early stage of breast cancer. This may be explained by the cancer stem cell hypothesis, which postulates that tumors are hierarchically organized and originated from a small subpopulation of cells, the so called 'cancer stem cells' (CSCs). These undifferentiated and mainly quiescent cells are posited to drive tumorigenesis through long term self renewal and the ability to differentiate into the tumor bulk population, contributing to tumor maintenance, metastasis, relapse and drug resistance. Nerve Growth Factor (NGF), the first discovered neurotrophic factor, has been shown to be synthesized and released by breast cancer cells, on which it acts as a potent activator of both the survival and proliferation. NGF exerts its effects through two membrane receptors: p75NTR, that belongs to the tumor necrosis factor (TNF)-receptor family and that it's the common receptor of all neurotrophines and pro-neurotrophines, and the tyrosine kinase receptor TrkA. Given the role of NGF in breast cancer development, the project aims to assess the potential involvement of NGF on breast CSCs biology, and to determine the underlying molecular mechanism. The study has been performed on the luminal MCF-7 and the basal-like MDA-MB-231 breast cancer cell lines. Cell culture in non-adherent conditions showed that both cell lines are able to form spheres for several generations. NGF does not modify the percentage of sphere forming units, which reflects indirectly the number of stem cells in the original culture. In contrast, NGF increased about 50% of mammosphere size upon 2 weeks of culturing. The increased sphere size resulted from enhanced proliferation of cells within spheres.
To further understand how NGF exerts this proliferative effect we first established MCF-7 and MDA-MB-231 cell lines overexpressing the receptors p75NTR or TrkA. We observed that the p75NTR overexpression per se increased the percentage of ALDEFLUOR positive population, although the involvement in NGF-mediated proliferation remains to be described. p75NTR is already considered as a marker for progenitor cells in melanoma, in esophageal and corneal epithelial cells, and this result suggests the involvement of the receptor in the activation of the breast cancer stem cells compartment. On the other hand, although TrkA overexpression per se did not seem to influence mammosphere formation, NGF strongly increased the number of sphere forming units in TrkA overexpressing cells. We conclude that NGF through its receptors seems to regulate the stem cell compartment either by the activation of quiescent cells or by increasing the size of the CSC compartment.

Poster Board Number: 2096

INVOLVEMENT OF OCT-4 IN HUMAN EPITHELIAL TUMORS

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Characterization of EWS-Oct-4 translocation fusion product in bone and soft-tissue tumors revealed a chimeric gene resulting from an in-frame fusion between EWS exons one to six and Oct-4 exons one to four. Recently, an alternative form of the fusion protein between the EWS and Oct-4 genes, named as EWS-Oct-4B, was reported in two types of epithelial tumors, a hidradenoma of the skin and a mucoepidermoid carcinoma of the salivary glands. As the N-terminal and POU domains of the EWS-Oct-4 and EWS-Oct-4B proteins are not structurally identical we decided to investigate the functional consequences of the EWS-Oct-4B fusion. We found that this new chimeric gene encodes a nuclear protein that binds DNA with the same sequence specificity as the parental Oct-4 protein, or the fusion EWS-Oct-4 protein. We show that the nuclear localization signal of EWS-Oct-4B is dependent on the POU DNA binding domain, and we identified a cluster of basic amino acids, 269RKRKR273, in the POU domain that specifically mediates the nuclear localization of EWS-Oct-4B. Comparison of the transactivation properties of EWS-Oct-4B and EWS-Oct-4 indicated that EWS-Oct-4B is a less potent transcriptional activator of a reporter construct carrying Oct-4 binding sites. Deletion analysis of the functional domains of EWS-Oct-4B revealed that the EWS N-terminal domain (NTD)B, POU, and C-terminal domain (CTD) are necessary for its full transactivation potential. Despite its reduced activity as a transcriptional activator, EWS-Oct-4B regulated the expression of fgf-4 (fibroblast growth factor-4) and nanog, which are potent mitogens, as well as Oct-4 downstream target genes whose promoters contain potential Oct-4 binding sites. Finally, ectopic expression of EWS-Oct-4B in Oct-4-null ZHBTc4 ES cells resulted in increased tumorigenic growth potential in nude mice. These results suggest that the oncogenic effect of the t(6;22) translocation is due to the EWS-Oct-4B chimeric protein and that alternative fusion of the EWS amino terminal domain to the Oct-4 DNA binding domain produces another transforming chimeric product in human epithelial tumors.

Poster Board Number: 2098

HORMONAL DEPRIVATION ENRICHES FOR MURINE UTERINE EPITHELIAL STEM CELLS WITH *IN VIVO* AND *IN VITRO* REGENERATIVE ACTIVITY

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Endometrial carcinoma, the most common gynecologic cancer in the U.S., is a hormonally sensitive tumor arising from the endometrial epithelia lining the uterine cavity. Despite an increase in both incidence and mortality in recent years, there is a dearth of meaningful basic and translational research looking at the pathogenesis of this disease. The cell of origin for endometrial carcinoma is unknown. We hypothesize that adult uterine epithelial stem cells may be the most efficient target for initiation of this tumor. Epithelial stem cells must exist in the endometrial lining. During reproductive years, the uterine lining sheds and re-grows on a monthly basis. With menopause the endometrium undergoes atrophy but retains the capacity to proliferate with the administration of estrogen hormone replacement therapy. These clinical observations suggest that uterine epithelial stem cells may be resilient to hormonal deprivation. The identity, location and characteristics of endometrial epithelial stem cells remain unknown. To identify these stem cells we have recently established *in vitro* and *in vivo* assays to measure growth and self-renewal capacity of isolated and dissociated uterine epithelial cells (UtE). Murine UtE single cells, isolated by FACS, grew as hollow spheres in a 3-dimensional matrix. The histology and marker expression profile of UtE spheres were similar to native endometrial epithelial glands. The number of spheres generated was linear with respect to input cells suggesting clonal growth. Clonality was further supported when only single color spheres were obtained from mixtures of GFP- and DsRed-labeled UtE cells. UtE spheres had the capacity to self-renew demonstrated by serial passaging

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of dissociated sphere cells. FACS sorted single UtE could also regenerate *in vivo* in combination with inductive stroma in the sub-renal capsule space and gave rise to functional endometrial-like glands. Titration experiments revealed that approximately 1 in 1000 UtE cells were capable of gland formation *in vivo*. Withdrawal of hormones from uterine epithelium, achieved surgically by ovariectomy, increased *in vitro* and *in vivo* regenerative activity 5-fold and 10-fold respectively. We demonstrate that uterine epithelial stem cells exist and are resilient to hormonal deprivation. Endometrial carcinoma is a disease commonly diagnosed in post menopausal women. Biologic hormonal deprivation with the onset of menopause may enrich for uterine epithelial stem cells, a potential target for cancer initiation. In future experiments, the cancer initiating capacity of uterine epithelium enriched for stem cells will be assessed using our *in vivo* regeneration system, previously shown to be an efficient model for studying endometrial cancer.

Poster Board Number: 2100

CISPLATIN INDUCED DNA DAMAGE INCREASES THE STEM LIKE CANCER CELLS IN HUMAN NON SMALL CELL LUNG CANCER

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Cancer stem cell hypothesis suggests that the side population of cancer cells exhibits stemness and drug resistant properties and mediates tumor initiation and tumor recurrence. Although the origin of cancer stem cells remains in debate, accumulating evidences demonstrates that the stem-like cancer cells can be induced by several cell stress, including inflammation, hypoxia, and DNA damage. Cisplatin, an anti-cancer drug used as first-line chemotherapy, induces apoptosis by causing DNA damage in lung cancer cells. However, cisplatin is not effective in non-small cell lung cancers (NSCLCs) because of the low response rate and high recurrence rate, although the mechanism is not clear. Thus, we hypothesize that cisplatin-induced DNA damage may induce stem-like cancer cells in NSCLC and increase the risks of drug resistance and tumor relapses. CD133 is discussed as a putative marker for cancer stem/tumor-initiating cell populations in epithelial tumors. So far, isolation of CD133+ side population from cancer cells depends on the antibody-based sorting techniques, which may not avoid of the non-specific binding effect. By transducing the human NSCLC cell lines, H460 and A549, with lentivirus carrying CD133 promoter-driven GFP reporter gene, 1.8% and 13.3% of GFP+ cells were distinguishable from H460 and A549 cell lines respectively. Comparing with GFP- cells, the GFP+ cells expressed higher protein and mRNA levels of CD133, also referred as CD133+ cells. These CD133+ cells were able to expand and to growth as tumor sphere in serum-free culture condition, suggesting the self-renewal capacity. In addition, the CD133+ cells also expressed the stemness genes, including Oct4, Nanog, and Sox2. The higher tumorigenic and metastatic potentials of the CD133+ cells were demonstrated by their greater soft agar-colony forming and migration ability. To test the effect of cisplatin on induction of stem-like cancer cells, H460, A549, and H1299 lung cancer cell lines were treated with 10 μM of cisplatin for 24h, which was sufficient to induce DNA damage. We found that treatment of cisplatin resulted in the increase of CD133+ cells from 1.8% to 49.5%, 13.3% to 32.8%, and 6.4% to 31.8% in H460, A549, and H1299 cell lines respectively. This increase of CD133+ cells did not due to the selection of the exiting CD133+ in the cultures, because the limited cell death was observed after treatment. Our ongoing study is focusing on the regulatory mechanisms that mediate the induction of stem-like cancer cells by cisplatin. Together, our study suggests that cisplatin is able to induce stem-like cancer cells in lung cancer, which may increase the risks of cross-drug resistance and tumor recurrence. The new compounds specifically targeting stem-like cancer cells are necessary to be included in the chemotherapy protocol for lung cancer.

Poster Board Number: 2102

MIR-145 ATTENUATION PROMOTES SELF-RENEWALING AND METASTATIC PROPERTY IN LUNG ADENOCARCINOMA

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MiR145, a tumor suppressive micro RNA, has been reported being involved in tumor growth and metastasis in several types of cancer, as well as regulating stemness genes in embryonic stem cells. However, the roles of miR145 in self-renewal and tumor-initiation of malignant lung adenocarcinoma (LAC) are still undetermined. In the current report, we showed that low level of miR145, accompanied with high level of Oct4 and Sox2, was found in metastatic and recurrent LAC patient specimens. We further demonstrated Oct4 and Sox2 as direct targets of miR145 in LAC. Forced overexpression of miR145 or silencing of Oct4/Sox2 in LAC-derived lung CD133+ cells decreased their stemness properties, repressed epithelial-mesenchymal transdifferentiation (EMT) through inhibiting mesenchymal-favored and enhancing epithelial-favored markers, and reduced primary cancer stem cell (CSC) population and *in vivo* tumor-initiating ability. Our results suggested miR145 as a potential switch regulating lung CSC-like properties, in which it negatively regulated EMT, a fundamental measurement of CSC characteristic, through inhibiting Oct4/Sox2/Fascin1. Targeting miR145 in LAC should be warranted in treating metastatic or recurrent LAC in the future.

Poster Board Number: 2104

IDENTIFICATION OF TUMORIGENIC CELLS IN KRASG12D INDUCED LUNG ADENOCARCINOMA

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Lung adenocarcinomas are one of the most lethal human malignancies that arise from the bronchiolar and alveolar epithelia. To investigate the cellular origin of this cancer, we report a novel strategy using FACS fractionation that could highly enrich bronchiolar ciliated, Clara, and alveolar type II cells in respective cell fractions to help dissect this unresolved issue. It was shown that bronchiolar Clara and alveolar type II cells were susceptible to oncogenic KrasG12D-induced transformation, whereas bronchiolar ciliated cells were not. EpCAM+MHCII- cells, which represent a bronchiolar origin, were more enriched in tumorigenic cells than EpCAM+MHCII+ cells, which represent an alveolar origin. In addition, secondary tumors derived from EpCAM+MHCII- cells showed phenotypic diversity of tumor locations and capable differentiation of an alveolar lineage compared to those derived from EpCAM+MHCII+ cells. High ERK1/2 activation and *in vitro* colony-forming ability were observed in EpCAM+MHCII- cells, in contrast to EpCAM+MHCII+ cells which had greater Sprouty-2 expression. These results suggest that bronchiolar Clara cells are the origin of cells and tumorigenesis for lung neoplasia after KrasG12D activation.

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A NOVEL PROTEIN IN HUMAN BRAIN TUMOR SUPPORTS HUMAN NEURAL STEM CELL MIGRATION THROUGH PI3-KINASE PATHWAY

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Many *in vivo* studies demonstrated a migration tendency of neural stem cell toward glioma. Stem cells from various lineages have become attractive vehicles to improve therapeutic gene delivery to brain tumors. However, little is known about the chemoattractive molecules to neural stem cells. To identify genes related to the tropism of human neural stem cells (NSCs) toward brain glioma, we compared the gene expression profile of human brain tumor with human normal brain tissues using micorarray analysis. Microarray analysis revealed that 150 secreted genes are up- or down-regulated in human brain tumor tissues, which were known to participate in tumor growth, inflammation, cell motility and apoptosis. The most differentially expressed secreted gene in this analysis is chemoattractant molecule 1 (CM1). Real-time PCR and immunocytochemical analysis detected CM1 gene expression in the brain tumor sample with a range of 3- to 7-fold elevation over normal brain. Then, *in vitro* migration assay with Boyden chamber assay showed that CM1 (~2-fold) attracted human NSCs more powerful than VEGF (1.25-fold), known chemoattract, do. While inhibition of ERK, Well known downstream signaling pathway, by PD98059 block CM1-meidated NSC migration by 25%, that of doublecortin (DCX) pathway, neural stem cell migration pathway, with roscovitin inhibited by half. These results demonstrate that CM1 participates in NSCs tropism toward brain tumor through ERK and DCX pathway.

Poster Board Number: 2108

HUMAN NEURAL STEM CELL-NANOPARTICLE HYBRIDS FOR TARGETED DELIVERY OF DIAGNOSTIC AND THERAPEUTIC COMPOUNDS TO INTRACRANIAL GLIOMA

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Brain tumors are difficult to treat because of our inability to accurately detect and selectively eliminate cancer cells. Despite the identification of effective diagnostic and tumor-toxic compounds, carriers are needed that selectively target these compounds to tumors so that healthy tissue remains undamaged. Neural stem cells (NSCs) exhibit tumor tropism and can provide the required tumor-specificity. Here we show that nanoparticles containing either small molecule chemotherapeutics or magnetic resonance imaging agents can be coupled to NSCs via a stable avidin-biotin linkage without impairing NSC tumor tropism. Using established methods, HB1.F3 NSCs were biotinylated by modifying cell-surface sialic acid residues. NSC viability was not affected by this process ($p>0.05$). Fluorescence activated cell sorting (FACS) results demonstrate biotinylation efficiencies of 85±9% when cells are labeled with FITC-conjugated avidin. Chromogenic and fluorescent immunostaining results confirm efficient NSC biotinylation. Biotinylated cells were incubated with streptavidin-conjugated nanoparticles (diameter=300 nm) which resulted in the stable addition of an average of 15 ± 8 nanoparticles/cell. A lower level of nanoparticle adsorption to unmodified control NSCs was also observed (2±1 nanoparticles/cell). NSC-nanoparticle hybrids maintained unimpaired *in vitro* tropism towards tumor conditioned media ($p>0.05$) as assessed using boyden-chamber chemotaxis assays. *In vivo* studies also demonstrate that human-nestin positive NSC-nanoparticle hybrids that were injected intravenously into mice with firefly luciferace-expressing intracranial GL261 glioma are able to cross the blood brain barrier and target brain tumors. Diagnostic and therapeutic efficacy studies for NSC-nanoparticle hybrids are underway. This research establishes proof-of-principle evidence that NSC-nanoparticle hybrids can deliver diagnostic and therapeutic payloads to tumors. This approach has great potential to improve the detection specificity of any diagnostic agent and the therapeutic index of any chemotherapeutic drug.

Poster Board Number: 2110

REPROGRAMMING MOUSE BRAIN TUMOUR STEM CELLS WITH DEFINED FACTORS

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The ability to reprogram somatic cells to generate induced pluripotent stem (iPS) cells has provided a tool for studying the epigenetic state of cells. This may be particularly interesting in the study of cancer, which results from a combination of genetic and epigenetic aberrations. Glioblastoma (GBM) is the most common and aggressive type of malignant brain tumour occurring in adults. The direct reprogramming of brain tumour stem cells (BTSCs) to generate iPS cells has not been reported to date, and may provide insights pertaining to the genetic and epigenetic aberrations occurring in brain cancer. We hypothesize that the epigenetic program of BTSCs can be reset by direct reprogramming with the Yamanaka Factors (Oct4, Klf4, Sox2, and c-Myc) to generate iPS cells, allowing us to study the role of epigenetics in glioma progression. We also hypothesize that neural stem cells (NSCs) generated from the tumour-derived iPS cells may have an altered tumourigenic potential. BTSCs derived from a CNS-specific p53-deficient mouse model of glioma were grown as adherent cell lines in serum-free media with EGF and FGF. These cells endogenously express Klf4, Sox2, and c-Myc, but fail to express pluripotency-associated genes, such as Nanog, Oct4, and Rex1. Mouse glioma BTSCs and normal NSCs were infected with retroviruses encoding Oct4, Klf4, Sox2, and c-Myc and cultured on feeders with serum and LIF to generate iPS cells. Ectopic expression of the four factors in BTSCs and normal NSCs generated colonies with typical iPS morphology. Colonies were sub-cloned and characterized by RT-PCR, which revealed the activation of endogenous expression of the pluripotency factors Nanog, Oct4, and Rex1 in reprogrammed clones. Dual inhibition (2i) of MAPK/Erk signaling and GSK3, which has been shown to promote full reprogramming, enhanced the expression of Nanog in a glioma-derived iPS cell line. Teratoma assays were carried out to assess the pluripotency status of the iPS cell lines *in vivo*, and intracranial injections were performed to determine the tumourigenicity of the iPS cell lines. This study will aid in elucidating the relative contribution of genetics and epigenetics to the neoplastic phenotype of glioma. By investigating the aberrant changes in the epigenetic program of BTSCs that promote tumourigenesis, we may identify novel genes or signaling pathways that are altered in glioma, which could be targeted by new chemotherapeutic agents interfering with chromatin modification.

Poster Board Number: 2112

LACTOBACILLUS CASEI INHIBIT THE GROWTH IN HUH7 CELL LINE

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Lactobacillus casei are known as lactic acid bacteria (LAB) that are widely used in dairy fermentations, and have many useful effects such as protec-

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tion, enhancement of biotherapeutic activities in our body. L. casei also have anti-cancer effect for proliferation in many cancers. However, there are not known to effect of L. casei in liver cancer. To investigate effect of L. casei about relationship with growth and cancer stem cell. Effect of growth and cancer stem cells observed in liver cancer cell line huh7. Effect of growth investigated through cell counting using trypan blue staining, and effect of cancer stem cells through analysis of side population cells (SP), which is known as cancer stem cells in liver cancer. To observe effect of L. casei about relationship with growth and SP cells in huh7 cells, lysate of L. casei treated to huh7 cells, investigated in growth rate, cell cycle, apoptosis, and Side Population(SP). L. casei induced 23% inhibition of growth compared untreated cells (control) to treated cells in huh7 cells. Inhibition of growth is caused by induction of cell cycle arrest and apoptosis. Huh7 cells increased 3% G2/M phase after lysate of L. casei treatment. Lysate of L. casei induced 5% cell death through Annexin V staining. In contrast to effect of growth, lysate of L.casei induced a 0.5% increase of SP cells. These results showed that L. casei induced inhibition of growth through induction of G2/M phase arrest and cell death but not target cancer stem cells. In conclusion, although L. casei is useful effect of inhibition of growth, these treated carefully in respect to targeting of cancer stem cells.

Poster Board Number: 2114

SSEA-4 EXPRESSION IS NOT ASSOCIATED WITH SIDE POPULATION OF HUH7 HEPATIC CARCINOMA CELLS

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Stage-specific embryonic antigen 4 (SSEA-4), one of markers of human embryonic stem cells, is known to be expressed in adult stem cells, such as mesenchymal stem cells, neural stem cells, and fetal liver progenitor cells. Although the expression of SSEA4 was recently detected in cancer cells and proposed as a marker of cancer stem cells, its role in liver cancer is largely unknown. In this study, we examined the expression of SSEA-4 in side population (SP) cells of a liver cancer cell line, huh7 cells and investigated its role in proliferation, drug resistance, invasion, and migration. Approximately 3% of the huh 7 cell population expressed SSEA-4, as determined by FACS analysis. Both SSEA4high and SSEA4low/- cells sorted from total huh7 cells maintained their phenotype in culture up to 30 days. Interestingly, side population cells were detected mainly in SSEA4low/- cells but not in SSEA4high cells. SSEA4high cells showed a decreased in proliferation by 24% compared to SSEA4 low/- cells. Moreover, the migrating ability of SSEA4high cells was significantly decreased compared to SSEA4low/- cells. Significant differences in invasive ability and drug resistance were not found between two cell populations. In conclusion, these results suggest that SSEA-4 may have a tumor suppressor effect and could be used as a potential indicator of liver cancer progression.

Poster Board Number: 2116

HDAC3 INDUCES EPITHELIAL-MESENCHYMAL TRANSITION AND STEM-LIKE PROPERTY IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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The epithelial-to-mesenchymal transition (EMT), one important process for cancer metastasis, which allow carcinoma cells for acquiring mesenchymal-like properties to promote cell migration and invasiveness. Recently, EMT also has been demonstrated to generate cells with stem-like properties in either human mammary epithelial cells or head and neck squamous cell carcinoma (HNSCC). But little is known about whether chromatin modification and modifier regulate EMT and EMT-induced tumor-initiating capability. Here, we show that histone deacetylase 3 (HDAC3), activated by hypoxia directly, is essential to induce EMT and metastasis phenotypes *in vitro* and *in vivo*. HDAC3 cooperates with EMT regulator-Snail and histone methyltransferase and causes specific histone modification changes, leading to repression of epithelial genes and activation of mesenchymal genes. We additionally demonstrate that overexpression of HDAC3 increases the subpopulation of HNSCC cells expressing putative tumour-initiating cell markers, suggesting the role of HDAC3 in EMT-induced tumor-initiating capability. These results suggest the important role of chromatin modifier in promotion of EMT and tumor-initiating capability and also highlight the potential use of epigenetic inhibitors in clinical treatment.

Poster Board Number: 2118

TARGETING OF HEAD AND NECK CANCER STEM CELLS USING SALINOMYCIN

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Cancer stem cells are believed to play a crucial role in cancer recurrence due to their resistance to conventional chemotherapy and capacity for self-renewal. Gupta and colleagues recently reported that salinomycin, a livestock antibiotic, selectively targets breast cancer stem cells. In our study we sought to determine the effect of salinomycin on head and neck squamous cell carcinoma (HNSCC) stem cells. We observed that salinomycin inhibits self-renewal capacity in HNSCC stem cells, as shown by a dose-dependent decrease in sphere formation. In addition, treatment with salinomycin resulted in a decrease in mRNA expression of the stem cell markers CD44 and BMI-1, both of which are critical for maintaining tumorigenicity in HNSCC. In combination with the chemotherapeutic agents cisplatin and doxorubicin, salinomycin synergistically killed HNSCC cancer stem cells more effectively compared to either drug alone, as demonstrated by MTS assays. High-throughput analysis revealed a set of differentially expressed microRNAs in salinomycin-treated HNSCC stem cells, warranting further investigation into the possibility of microRNA-mediated pathways that are regulated by salinomycin. Taken together, our findings indicate promise for using salinomycin as a novel treatment for HNSCC.

Poster Board Number: 2120

CANCER STEM CELL SELF-RENEWAL IS ADDICTED TO MYC ONCOGENE

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Background: Cancer cells are addicted to oncogene for survival and growth. Whether, cancer stem cells (CSCs) are also addicted to oncogene for growth and self-renewal is not yet known. Here, we used a conditional MYC model of murine T-cell lymphoma to systematically relate the dependency of cancer stem cells to MYC oncogene for self-renewal and tumorigenicity. Methods: The primary Myc-induced T- cell lymphoma tumors were dissociated and flow-cytometry sorting was performed to isolate CSCs by flow cytometry. The sorted cells were injected to congenic mice intravenously and tumor formation was monitored. Results: We isolated a rare fraction (~ 0.01%) of CSC cells using flow-cytometry for SP-sorting of primary tumors. While only 100 cells of CSC fraction developed disseminated lymphoma in congenic mice (intravenous injection), 106 of the non-sorting primary cells formed only rare tumors (1/25 mice at 6 months). When, the CSC fraction was re-injected to congenic mice, rapidly growing disseminated tumors were found in liver, spleen and mesenteric lymph nodes suggesting that the rare fraction suggesting they were maintained in the secondary tumors. *In vitro*

clonogenic assay using methylcellulose media demonstrated that the CSC fraction was 5x103 fold clonogenic (p<0.001). Recent studies suggest that MYC is involved in inducing an embryonic stem cell (ESC)- like gene signature in aggressive cancer. We found that inhibition of MYC expression both *In vitro* and *in vivo* appeared to suppress the CSC fraction. Conclusion: We conclude that CSCs are dependent on MYC oncogene for both growth and self-renewal.

Poster Board Number: 2122

LY294002 INDUCES APOPTOSIS AND NEURONAL DIFFERENTIATION IN MDB CANCER STEM CELLS

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Medulloblastoma (MDB) is the most common cancerous brain malignancy of childhood, with only a 60% of 5-year overall survival. In the last years remarkable advances have been made trying to understand the pathogenesis and genetic alterations involved in MDB, although outcome for this type of tumour is still poor. The PTEN/PI3K/Akt/mTOR signalling pathway, crucial in many aspects of cell growth, survival and proliferation and involved in the pathogenesis of different human malignancies, could be implicated in the molecular pathogenesis of MDB too and represents a novel therapeutic target in this disease. Moreover it is currently thought that MDB arises from cerebellar stem cells that have been prevented from dividing and differentiating into normal cell and that are considered responsible for tumour relapse. We previously found that the treatment with LY294002, a specific inhibitor of the PI3K/Akt/mTOR signaling pathway, promoted a block of cell cycle at G1 phase, followed by apoptosis, in MDB cell lines in a time dependent manner. Moreover, LY294002 treated cells formed less colonies than control. The Side Population (SP) cells, isolated by FACS, displayed a higher *in vitro* clonogenicity in soft agar compared to non-SP cells in all MDB cell lines tested and, importantly, SP cell fraction resulted to be more sensitive to LY294002 treatment. Here we have investigated the *in vitro* effect of the PI3K specific inhibitor, in primary MDB-derived cells and we found that LY294002 treatment impair cell proliferation, with a block of cell cycle at G1 phase followed by apoptosis. We observed that reduced cell proliferation, after LY294002 treatment, was characterized by neuronal differentiation, as shown by the increase of β-III-tubulin+ and the concomitant decrease of Nestin+ MDB-derived cells. We also analyzed the expression of CD133, a stem cell marker, and we found that LY294002 treatment caused a decrease in the percentage of CD133 positive cells in a time dependent manner and that CD133 negative cells are not affected by the treatment. Moreover we found that sorted CD133+ cells were more sensitive, compared to CD133- cells, to LY294002 treatment, as shown by the increase of Annexin+ cells. These preliminary results suggest that PTEN/PI3K/Akt/mTOR signaling pathway could be critical for MDB stem-like cells maintenance and that it could be selectively targeted for inhibiting cancer stem-like cells for improved treatment.

Poster Board Number: 2124

TARGETING SIDE POPULATION CELLS IN AGGRESSIVE FIBROMATOSIS

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Aggressive fibromatosis (AF) is a locally invasive, benign soft-tissue tumor, composed of fibroblast-like cells that arise from musculoskeletal structures. Despite their tendency to infiltrate into surrounding normal tissues, they do not metastasize to distant sites. These lesions can occur either sporadically or as part of preneoplastic conditions. AF tumors harbor mutations activating beta-catenin mediated signaling, and genetically modified mice that exhibit beta-catenin protein elevation develop AF tumors. Evidence from mice shows that AF is derived from mesenchymal progenitor cells containing a mutation activating beta-catenin mediated signaling. Mesenchymal neoplasms contain a subpopulation of cells that exclude Hoechst 33342 dye (Side Population, or SP cells) that are enriched for tumor initiating potential, and as such have stem cell like properties. Using expression profiling, we examined SP and non-SP cells from human AF tumors. Analysis of the expression profiles using the ingenuity expression analysis program showed that the Hedgehog (Hh) signaling pathway is differently regulated in side population (SP) compared to non-side population (NSP) cells from human AF samples. Since the Hh signaling can maintain mesenchymal progenitors in a less differentiated state, we examined the role of Hh signaling blockade in this tumor type. Apc1638N mice harbor a germ line mutation in the Apc gene, and develop large numbers of AF tumors. We treated Apc1638N mice with triparanol (which blocks Hh signaling), or a carrier as a control. We found that triparanol treatment decreased Hh signaling in the tumors, and resulted in a 25% decrease in the tumor volume in treated mice compared to control mice (P<0.013). This data shows that targeting Hh signaling in AF has the potential to developed into a therapeutic approach to treating AF, and raises the possibility that targeting SP cells could be used to treat select neoplasms.

Poster Board Number: 2126

THERAPEUTIC POTENTIAL OF SILYMARIN IN CANCER STEM CELLS

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Silymarin, a flavonolignan complex isolated from milk thistle (Silybum marianum L. Gaertn), has a strong antioxidant activity and exhibits anti-carcinogenic, anti-inflammatory, and cytoprotective effects. Although the anti-carcinogenic effects of silymarin on various types of cancers have been previously documented, the effect of silymarin on cancer stem cells (CSCs) is largely unknown. Recently, several studies have been shown that a growing tumor is a heterogeneous mix of mostly differentiated cancer cells and CSCs, a rare population that have stem cell-like properties and exhibits more resistance to conventional therapies. Since restricted oxygen conditions increase the CSC fraction and promote acquisition of a stem-like state, CSCs are critically dependant on the hypoxia-inducible factors (HIFs) for survival, self-renewal, and tumor growth. Mammalian target of Rapamycin (mTOR) is a regulator of HIF expression and activity; therefore, the inhibition of HIF-mediated gene expression is considered to be related to the antitumor activity of mTOR inhibitors in some cancers. Moreover, it has been shown that mTOR-inhibitors suppress the expression of CD133, a stemness marker of CSCs, suggesting a role for mTOR in the regulation of CD133 expression. Taking into consideration that silibinin, the major active constituent of silymarin, was found to inhibit HIF-1α and mTOR, silymarin could be considered as a novel potential therapeutic strategy targeting CSCs. Novel mechanisms of anti-carcinogenic effects of silymarin on CSCs is currently under investigation in our institution.

Poster Board Number: 2128

A NOVEL PLURIPOTENCY-RELATED ONCOGENE NETWORK IN HESC AND IPS CELLS

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There are many important links between tumorigenesis and pluripotency. For example, the genes used to produce induced pluripotent stem cells (IPSC) are strongly associated with cancer and highly expressed in human

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embryonic stem cells (hESC). hESC and IPSC both can robustly form teratoma as well. To test the notion that pluripotency-related genes may have key roles in tumorigenesis and search for stem cell-related oncogenes that might be important in teratoma formation, here we conducted an expression screen of a novel retroviral hESC cDNA library that we produced. When we transduced fibroblasts with the hESC cDNA library, we found remarkably robust cancer cell formation, indicating that dozens of genes that are highly expressed in hESC are putative oncogenes that can readily drive cellular transformation. All viral cDNAs from cancer colonies that we have tested, have been validated as powerful single-hit oncogenes. After sequencing to identify the cDNAs acting as oncogenes, we found known oncogenes but also many novel oncogenes. Gene ontology analysis indicated that many of these new oncogenes encode proteins that interact together in a functional network. Thus, from this screen we have discovered a network of novel stem cell-related oncogenes that control cellular metabolism and cell cycle. At the center of this network is a single core pluripotency-related transcription factor also identified in our screen, but not previously linked to cancer. This gene is highly expressed in germ cell tumors such as teratoma and in other cancers including leukemias. Strikingly the transcription factor it encodes regulates expression of many of the other oncogenes that we identified. Our findings have critical implications for understanding stem cell biology and tumorigenesis as well as for the development of safe regenerative medicine therapies based on IPSC and hESC.

Poster Board Number: 2130

P27 TUMOR SUPPRESSOR REPRESSES SOX2 EXPRESSION

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p27Kip1 is an inhibitor of the cell cycle dependent kinases (Cdkn1b). Mice deficient for p27 develop pituitary tumors, exhibit organ hyperplasia resulting in increased body size, and retinal dysplasia. Decreased expression of p27 is linked to multiple human cancers, particularly lung cancer. On the other hand, Sox2 is one of the core ES transcription factors. Interestingly, heterozygous loss of Sox2 in mice results in reduced body size, atrophic retina, and hypopituitarism. More recently, overexpression of Sox2 has been associated with lung cancer in both mice and human. This reversed phenotype prompted us to further investigate the potential interplay between p27 and Sox2 during development, cancer and reprogramming. Here, we report that p27 deficiency causes an increase in the expression of Sox2 and in the number of Sox2 positive cells in mice. Furthermore, the combination of p27 deficiency with Sox2 heterozygosity largely rescued all the phenotypic defects observed in either single p27 or Sox2 deficient mice, thus normalizing body size, retinal organization, and pituitary size and development. To further elucidate the molecular mechanisms connecting p27 and Sox2, we observed that during *in vitro* ES differentiation, p27 directly binds to Sox2 promoter and contributes to its transcriptional repression. Furthermore, retinoic acid-induced differentiation of p27-/- iPSCs failed to reduce Sox2 expression as efficiently as in wild type iPSCs. Finally, mouse embryo fibroblasts derived from p27-/- mice can be reprogrammed with only Klf4 and Oct4. All together, we conclude that p27, a cell cycle regulator, directly regulates Sox2, a stemness gene. This regulation is important for differentiation, development and cancer.

Poster Board Number: 2132

CELL REPROGRAMMING ALLEVIATES EXPRESSION ABERRATIONS OF SKI AND PRDM16 IN GASTRIC CANCER CELLS

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Both the origin of cancer and the reprogramming process are thought to involve important and widespread epigenetic changes inside cells. We investigated the possibility of carcinogenic pathway suppression through reprogramming gastric cancer cells. Two specific genes, SKI and PRDM16 (also known as MEL1), have been shown to be aberrantly over-expressed in gastric cancer cells. These two genes interact with Smad3 and Smad4 to restrict the transforming growth factor (TGF)-β signaling pathways in gastric cancer cells, ultimately causing malignancies. Through the process of induced pluripotent stem cell (iPSC) reprogramming, we have been able to reduce the expression of these genes, thereby reducing the cancerous traits of these cells. We reprogrammed a gastric cancer line MKN28 using a retroviral system of the four transcription factors Oct4, Sox2, Klf4, and c-Myc. A unique protocol was developed to reprogram this media-sensitive cell type. The reprogrammed cancer cells exhibited iPSC morphology of tightly packed colonies composed of small cobblestone-shaped cells that possess a high nucleus-to-cytoplasm ratio. Immunostaining assays showed that these colonies expressed the pluripotency markers alkaline phosphatase, SSEA4, Tra-1-60, and Nanog while the parental MKN28 cells did not. Quantitative RT-PCR results showed the increasing endogenous expressions of Nanog, Cripto and the four transcription factors while the retroviral transgenes were silenced, indicating a reprogrammed, pluripotent state. After validating the pluripotency of reprogrammed MKN28 cells, we compared the expression of SKI and PRDM16 of the parental MKN28 gastric cancer cells to the reprogrammed cells through quantitative RT-PCR and immunoassaying. qRT-PCR results showed that the reprogrammed cancer cells have a significantly reduced gene expression of SKI and PRDM16 when compared to MKN28 parental cells. Our immunostaining results confirmed the qRT-PCR results, as they showed higher expression of SKI in MKN28 parent cells than in the reprogrammed cells. These results demonstrate that the iPSC reprogramming process alleviates the over-expression of onco-proteins and alters TGF-β signaling pathways, suggesting possible effects of reprogramming on reducing malignant characteristics of cancer cells.

Poster Board Number: 2134

FGF2 MEDIATES RAPID DNA REPAIR IN SP CARCINOMA CELLS

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Cancer stem cells can resist to anticancer therapy through various mechanisms, including overexpression of endogenous factors or extracellular proteins of the SC niche. The secreted form of the growth factor FGF2 can be a component of the tumor cell micro-environment, originating from the tumor stroma or from the tumor cells. This factor has also a nuclear form, whose function is largely unknown. As we demonstrated that the secreted form of FGF2 can be a regulator of DNA repair in normal epidermal stem cells through the canonical signaling pathway using FGF2 receptor and MEK kinases, we here address such mechanisms in carcinoma cells. We isolated the SP and non-SP populations (NSP) from the A431 carcinoma cell line using flow cytometry, on the basis of Hoechst dye exclusion. This SP cells (1-2 %

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of the total population), has been shown to be more tumorigenic than NSP cells. Investigations of radiation-induced DNA damage and repair using the alkaline comet assay revealed that the damage was repaired more rapidly in SP than in NSP cells. When the FGF2 signaling pathway was blocked using the MEK1 inhibitor UO126, the irradiated SP cells lost their capacity of rapid DNA repair. Moreover, addition of exogenous FGF2 in the growth medium of NSP before irradiation increased the repair of radiation-induced damage. These data show that FGF2 can protect carcinoma cells by activating stress-induced DNA repair. They support the hypothesis that targeting FGF2 signaling pathway could potentiate carcinoma radiotherapy. To better understand the origin of FGF2, the expression of this cytokine was characterized in the two carcinoma cell populations. NSP cells expressed low levels of intra and extra cellular forms of FGF2, both before and after irradiation. The possible effects of FGF2 on NSP in a tumor could then rely on a stromal origin, and use the canonical signaling pathway through the FGF2 receptor. SP cells expressed constitutively more intracellular and secreted FGF2 than NSP cells, before irradiation. We propose that the more tumorigenic cellular fraction in the carcinoma cell line is primed to a more rapid response to radiation by this endogenous high level of FGF2. We postulate that a high content in the nuclear form of FGF2 plays an important role in their rapid DNA repair, and that downregulating endogenous FGF2 could be a way to radiosensitize cancer stem cells in carcinoma.

Poster Board Number: 2136

HYPOXIA ENHANCES TUMOR STEMNESS OF A RARE FRACTION OF ES CELLS BY UPREGULATING HIF-2ALPHA

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Background: A long-standing question in stem cell biology has been whether transient exposure of stem cells to the area of injury is sufficient enough for malignant transformation. The site of injury/inflammation is characterized by hypoxia and ROS (reactive oxygen species). Stem cells, when exposed to such stressful microenvironment may switch to an unstable but highly functional, highly undifferentiated state, the "enhanced stemness" state by upregulating HIF-2α, which in turn may upregulate Oct-4, Nanog and MDM2 to switch the stem cell state to a tumor stemness state. We investigated this possibility by exposing hESCs to extreme hypoxia. Methods: Human ES cells (hESCs-BGO1v) were exposed to an extreme environment of *in vitro* hypoxia/oxidative stress that mimics *in vivo* injured microenvironment. FACS sorted surviving SSEA3 and ABCG2 fraction (3 days post hypoxia) was subjected to QPCR (real time PCR) and In-cell western blot assay to study the expression of enhanced stemness genes. HIF-2α was inhibited by siRNA knockdown. Results: FACS analysis revealed that BGO1v cells contain a rare fraction (about ~0.1%) expressing both SSEA3 and ABCG2. When exposed to hypoxia, a small fraction of cells, SSEA3+/ABCG2+, about ~4% survived and retained the undifferentiated state even after 3 weeks of culture, whereas the SSEA3+/ABCG2- underwent differentiation and apoptosis. When the surviving SSEA3+/ABCG2+ cells were expanded for another three weeks, an EC like clone (resembling a Tera-2 like clone in the cell culture plate) was obtained. This post-hypoxia clone (denoted henceforth as ESt; t denoting tumor) demonstrated higher levels of Nanog, SSEA3, HIF-2α, N-MYC and MDM2 and ABCG2 than the parental BGO1v cells. The ESt fraction was characterized by significantly higher clonogenic activity than the pre-hypoxia SSEA3+/ABCG2+ fraction. Most importantly, the SSEA3+/ABCG2+ cells, FACS sorted from the Est clone, required only one fifth of the number of cells required to form a rapidly growing teratoma in nude mice compared to the pre-hypoxia SSEA3+/ABCG2+ fraction (10,000 cells versus 50,000 cells respectively). The dissociated teratoma obtained from the ESt clone showed the presence of ~1% of SSEA3+/ABCG2+ve cells, which when re-injected i.p. formed a rapidly growing secondary teratoma suggesting an EC like teratocarcinoma forming activity of the ESt cells. The siRNA knockdown of HIF-2α induced differentiation and apoptosis of the SSEA3+/ABCG2+ fraction during exposure to hypoxia. Conclusion: We conclude that only a rare fraction of highly undifferentiated hESCs could achieve this "enhanced stemness" state during exposure to hypoxia and oxidative stress, whereas the remaining cells either undergo apoptosis or differentiation.. Furthermore, on rare occasion, the "enhanced stemness" state is stabilized into a "tumor stemness" state.

Poster Board Number: 2138

ISOLATION AND CHARACTERISATION OF LYMPHOMA INITIATING CELLS

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Haematological malignancies comprise a variety of functionally heterogeneous cells thought to arise from discreet cellular subsets, and are responsible for tumour maintenance and metastasis. These discrete cellular subsets are termed Tumour-Initiating Cells (TICs) and are so called because of their functional likeness to tissue-specific stem cells; they are able to self-renew, survive for the long term and give rise to the bulk of the tissue/tumour population and yet are relatively quiescent. Such cells have been described for a number of malignancies and are deemed responsible for continued tumour growth and propagation as well as for disease relapse and in some cases metastatic growth. It has yet to be determined whether TICs exist in Non-Hodgkin lymphoma (NHL), a heterogeneous disease affecting the cells of the immune system. There are greater than 30 sub-types of NHL and our initial focus has been on the Anaplastic Large Cell Lymphoma (ALCL) and Peripheral T-cell Lymphoma-Not Otherwise Specified (PTCL-NOS) categories, both representing mature T-cell malignancies. In this study, we provide evidence for the presence of a distinct sub-population of cells with stem-like properties within the bulk tumour population. We have employed the Side Population (SP) technique to identify cells with enhanced drug efflux capacity, an inherent property of stem cells. We show that a distinct population of SP cells exist in ALCL cell lines and that this population contains cells positive for the CD117 or c-kit (2-5%) cell surface marker commonly associated with haematopoietic stem cells (HSCs). Importantly, cells expressing c-kit are absent in the main population (MP; cells that are not detected as SP cells). Furthermore, in-vitro differentiation assays show that the isolated SP population can differentiate to give rise to more SP cells as well as the bulk differentiated tumour population. Conversely, the MP cells do not form new SP cells and fail to propagate. Real-Time quantitative PCR (qPCR) on SP and MP showed increased expression of ABCG2 and Oct4 transcription factors in SP cells contributing to its defense mechanism and self-renewal ability respectively. We have also identified a Verapamil-sensitive SP population in primary human ALCL and PTCL-NOS primary patient tumours and furthermore we demonstrate that 2-3% of these cells likewise express c-kit on their surface. This primary cell population, from ALCL and PTCL-NOS patients, is reproduced on transplantation of whole tumour cell populations into immunodeficient mice and furthermore when c-kit+ cells alone are transplanted into mice, they propagate to produce tumours representative of the primary sample whereas c-kit depleted tumour cells do not. Hence, we propose that at least some NHL may constitute a small population of TICs. Specifically, ALCL constitutes a small population of c-kit-positive cells which display properties of TICs.

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Poster Board Number: 2140

DIFFERENTIAL EXPRESSION OF CELL ADHESION MOLECULES IN RETINOBLASTOMA

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Purpose: Adhesion molecules, such as MUC-1, CD24 and CD44 play important roles in intercellular signaling in cancer, particularly in the context of cancer stem cells. The purpose of this study was to examine the expression of these molecules in retinoblastoma (RB) to provide insights into cell adhesion and potential mechanisms of metastasis. Methods: RB143, a short-term, low passage human RB cell line and WERI-RB27, a long-term, high passage human RB cell line, were analyzed for expression of adhesion molecules MUC-1, CD44 and CD24 by flow cytometry. Results: WERI-RB27 cells expressed neither CD44 nor CD24, but did contain a small percentage of MUC-1+ cells (13% positive). In contrast, virtually all RB143 cells were CD24+ (100% positive), while a small percentage of cells were MUC-1+ (16% positive). CD44, a major E-selectin ligand, was also not detected in RB143 cells. Conclusions: WERI-RB27 cells and RB143 cells differ greatly in the percentage of CD24+ cells, but exhibit similar numbers of MUC-1+ cells (13-16%). Both cell lines lack CD44 immunoreactivity. Based on these results and our previous studies, retinoblastoma cells appear to lack the CD44-E-selectin metastatic adhesion pathways and may rely on alternate mechanisms, such as ICAM-1 to accomplish metastatic spread and extravasation into target tissues.

Poster Board Number: 2142

ONCOGENES INDUCE STEM CELL LIKE PROPERTIES IN THYMOCYTE

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Thymocytes are devoid of self-renewal activity and long term thymic output in T lymphocytes requires input from hematopoietic stem cells. We now show that DN3 thymocytes are the target cells of transformation by the SCL-LMO1 oncogenes in T-cell acute lymphoblastic leukemia. Furthermore, ectopic expression of these oncogenes induces aberrant self-renewal in DN3 thymocytes which acquire thymic engraftment capacities when transplanted during the pre-leukemic phase. Remarkably, genetic manipulation of this stem cell-like activity during the pre-leukemic phase supports the view that aberrant self-renewal determines the penetrance and the aggressiveness of the disease. In addition, we provide genetic evidence that enhanced self-renewal was not due to a suppression of differentiation, since oncogene-induced aberrant self-renewal is independent of the Cd3 gene which is required for thymocyte differentiation. Rather, SCL-LMO1 upregulate a set of stem cell genes and challenge this limit in thymocytes. We therefore conclude that oncogene-induced aberrant self-renewal in thymocytes represent an important initiating event in T-ALL.

Poster Board Number: 2144

RESIDUAL EMBRYONIC CELLS AND THE ORIGIN OF A BARRETT'S-LIKE METAPLASIA

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Barrett's esophagus is an intestine-like metaplasia and precursor of esophageal adenocarcinoma. Triggered by gastroesophageal reflux disease, the origin of this metaplasia remains unknown. p63-deficient mice, which lack squamous epithelia, may model acid-reflux damage. We show here that p63 null embryos rapidly develop intestine-like metaplasia with gene expression profiles similar to Barrett's metaplasia. We track its source to a unique embryonic epithelium that is normally undermined and replaced by p63-expressing cells. Significantly, we show that a discrete population of these embryonic cells persists in adult mice at the squamocolumnar junction, the source of Barrett's metaplasia in humans. Upon programmed damage to the squamous epithelium, we show that these embryonic cells migrate towards and under adjacent, specialized squamous cells in a process that may recapitulate early Barrett's. Our findings suggest that particular precancerous lesions, such as Barrett's, derive not from genetic alterations but from competitive interactions between cell lineages driven by opportunity.

Poster Board Number: 2146

INVESTIGATION OF SOX2 INTERACTING PARTNERS IN EMBRYONAL CARCINOMA STEM CELL LINE, NTERA2

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Much attention has been paid to embryonic and induced pluripotent stem cells because of their great therapeutic potential in regenerative medicine. Identifying underlying mechanism of their properties especially self-renewal and pluripotency may lead to their reliable application in therapy and alternative ways of reprogramming. As it has been shown, protein interaction networks of transcription factors such as Oct4, Sox2 and Nanog and also epigenetic mechanisms are playing important role in establishment and maintenance of pluripotency, in this study we aim to investigate interacting partners of SOX2 in NTERA-2 cell line as a convenient model pertinent to human pluripotent stem cells. A combination of affinity purification of tagged SOX2 and LC-MS/MS were used for isolation and characterization of interacting proteins. The presence of factors such as XRCC6, HNRNPC and SET links the pluripotency to DNA replication, recombination, repair and promoter maintenance, RNA processing and epigenetic mechanisms, respectively.

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CXCR6, A NEWLY DEFINED BIOMARKER OF TISSUE SPECIFIC STEM CELL ASYMMETRIC SELF RENEWAL, IDENTIFIES MORE AGGRESSIVE HUMAN MELANOMA CANCER STEM CELLS

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The majority of human cancer cells are the clonal progeny of an initiating variant tissue cell. Recent investigations of a variety of tumor types have shown that phenotypically identifiable and isolable subfractions of cells possess the tumor-forming ability. This feature of rare cells with tumor-forming potential dividing to produce cells with limited or no tumor forming potential is the basis for the cancer stem cell paradigm. Cancer stem cell research revived a longer-standing idea that many tumors (though not all) were likely to be the spawn of mutated tissue-specific stem cells. We will present the first phenotypic evidence to support this concept. Another important point that will be discussed is the identification of a more aggressive subpopulation ABCG2/CXCR6+ in melanoma. CXCR6 is a new biomarker for tissue-specific cell asymmetric self-renewal. The association of more aggressive tumor phenotype with asymmetric self-renewal phenotype reveals a previously unrecognized aspect of tumor cell physiology. Namely, the retention of some tissue-specific stem cell attributes, like the ability to preserve self-phenotype, impacts the natural history of human tumor development. Knowledge of this new aspect of tumor progression may provide new targets for cancer prevention and treatment.

Poster Board Number: 2150

IDENTIFICATION OF ECCRINE GLAND MELANOCYTE STEM CELLS IN MOUSE ACRAL SKIN AS A POTENTIAL SOURCE OF ACRAL MELANOMA

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Acral volar skin is unique in that it contains (abundant) eccrine glands instead of hair follicles as the main skin appendage and is minimally exposed to UV sunlight. Acral melanoma is the most prevalent subtype of melanoma in the non-Caucasian population. The preferential proliferation of early acral melanoma cells along and around epidermal eccrine ducts has indicated a close association between early acral melanoma *in situ* and eccrine glands. However, neither the presence of melanocytic cells in the eccrine glands nor the precise origin of these melanoma cells has been identified to date. Here, we report the identification of eccrine gland melanocyte stem cells in mouse acral skin. We found that unpigmented melanocytic cells reside in the secretory portion of eccrine glands using lineage-tagged H2B-GFP reporter mice and have characterized the development and maintenance of this population in adult mouse skin. These melanoblasts are normally kept in an immature and slow-cycling state but are able to self-renew and provide transit amplifying progeny which migrates upward toward the epidermis concurrent with the synthesis and deposition of melanin pigment in response to stresses including ionizing irradiation. These findings indicate that this population possesses several features consistent with adult stem cells. The existence of an acral melanocyte stem cell population within the eccrine gland might explain the preferential proliferation of early acral melanoma cells around eccrine glands during the process of melanomagenesis.

GERMLINE CELLS

Poster Board Number: 2152

IMPROVED METHODS TO ISOLATE AND CULTURE VSEL STEM CELLS FROM HUMANS

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Very small embryonic-like (VSEL) stem cells have been isolated from human umbilical cord blood, murine and human adult peripheral blood and tissues. These cells uniformly express proteins characteristic of embryonic and induced pluripotent stem cells, have a high nuclear to cytoplasm ratio, and predominance of euchromatin. Murine VESL are capable of differentiation into endoderm, mesoderm and ectoderm cells, and human-derived VESLs are at least capable of neuronal differentiation. VSEL cells do not express markers of hematopoietic stem cells or possess re-populating capabilities of hematopoietic stem cells. The function of these cells in mammals is not well understood, but a role in regeneration is suggested by the finding that in humans the peripheral blood population fraction expands following myocardial infarction and stroke, and enhanced cardiac recovery from MI in a mouse model. We will report on a method to isolate VSEL stem cells from human non-CSF mobilized blood that yields 4-7 cells per uL of whole blood with 99% purity. This yield is ~10 times higher than other published methods and indicates that VSELs are more common than previously thought. Methods to culture human VSEL stem cells over extended periods have not been described. We report a method to propagate human peripheral blood VESL stem cells in culture. After six months VESL stem cells cultured by our methods continue express a similar profile of pluripotency markers as determined by a TaqMan pluripotency array and are phenotypically similar to VSEL stem cells harvested immediately from blood. These methods provide new research opportunities with VSEL stem cells.

Poster Board Number: 2154

PROSPECTIVE ISOLATION AND ENRICHMENT OF HUMAN SPERMATOGONIAL STEM CELLS, ENDOTHELIAL PROGENITOR CELLS AND TESTICULAR STROMAL CELLS FROM ADULT TESTIS

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Tissue-specific stem cells are known to possess therapeutic potential in regenerative medicine. Several markers are described to isolate hematopoietic and mesenchymal stem cells. Little is known, however, about the marker profile of human spermatogonial stem cells (SSCs). The main reasons are the difficult availability of tissue and the small number of SSCs that is present in testis. Markers currently used for isolation of human SSCs like CD49f and CD9 are not highly specific. Recent studies have shown that SSCs express CD49f. In the search of additional antigens, which are co-expressed on subpopulations of CD49f+ cells, we prepared single cell suspensions derived from testicular biopsies and screened a large panel of antibodies against surface antigens for their reactivity with CD49f+ cells. Flow cytometry

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analysis revealed that antibodies against CD49a and CD144 are suitable to further fractionate the CD49f+ population. Multicolor cell staining and FACsorting of testicular cells revealed that SSCs were exclusively found in CD49f+CD49a-CD144- population, as determined by colony forming assays and real-time qPCR using BioMark Dynamic Array. In contrast, testicular stromal cells (TSCs) were enriched in the CD49f+CD49a+CD144- subset and endothelial progenitor cells (EPCs) in CD49f+CD49a+CD144+ subset, which was verified by the specific expression of key markers defining mesenchymal (CD49a, CD73, CD90, CD105, CD140b, CD166) and endothelial cells (CD31, CD34, CD45, CD105, CD144, CD146, CD309), respectively. In conclusion, we identified a novel marker combination, which is suitable for the simultaneous isolation of SSCs, TSCs and EPCs from adult human testis.

Poster Board Number: 2156

CONTRIBUTION OF SPECIFIC TRANSCRIPTION FACTORS IN THE REPROGRAMMING OF MOUSE SOMATIC CELLS TOWARDS SPERMATOGONIAL STEM CELLS

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Studies over the past 15 years have culminated in the demonstration that we can isolate spermatogonial stem cells (SSCs) from the testes of several mammalian species, culture them *in vitro*, and in the mouse, transplant them to a germ cell depleted testis where they are capable of colonizing and producing mature functional sperm. Though SSCs can be enriched from the general 'germ stem' (GS) population in the testis, molecular characterizations that uniquely distinguish SSCs from any other stem cell type in the body have yet to be determined. Further, how SSCs arise from more primitive gonocytes remain to be fully elucidated. It is becoming clear that the larger GS population is characteristically and functionally heterogeneous and that the percentage of GS cells capable of colonizing a germ cell depleted testis in functional transplantation assays is very low. In an effort to further characterize spermatogonial stem cell differentiation we are forcing expression of specific SSC transcription factors to covert somatic cells to spermatogonial stem cells. The selected transcription factors were previously defined as important for SSC establishment or maintenance. cDNAs from a dozen factors were sub-cloned to lentiviral vectors for transduction to mouse embryonic fibroblasts (MEFs), and transduced and non-transduced MEFs were maintained in media that supports long-term culture of SSCs. RNA was collected from both transduced cells and non-transduced controls every 7 days for a period of 4 weeks to assess changes in gene expression by real time q-PCR. At 4 weeks, MEFs transduced with the SSC transcription factors showed an up-regulation in endogenous expression of stem cell specific genes such as Nanog and Pou5f1, as well as in germ cell specific genes including Plzf, Kit, Tex15 and Thy1 compared to non-transduced controls; an expression pattern consistent with that seen in SSCs *in vivo*. Ongoing experiments to refine our list of transcription factors will help us identify novel factors involved in the SSC transcriptional network and to define a collection of transcription factors, which could reprogram a somatic cell towards an SSC. Successfully generating an SSC *in vitro* would be a major advancement in the treatment of male infertility, enabling the generation of patient-specific SSCs to extend treatment options for men undergoing radiation or other cancer treatments, which ablate the germ cell populations in the testis.

Poster Board Number: 2158

THE INDUCTION OF DIFFERENTIATION OF MOUSE SPERMATOGONIAL STEM CELLS BY RETINOIC ACID

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Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis. SSCs self-renew for a lifetime and produce progenitor spermatogonia that ultimately transform into sperm. In recent years, several growth factors have been identified that promote SSC self-renewal and knowledge regarding the mechanism of self-renewal has been accumulating steadily. In contrast, the mechanism of SSC differentiation has remained elusive. Since the fundamental function of SSCs is to support sperm generation, i.e. differentiation, this lack of knowledge is a critical problem for understanding SSC biology and for the clinical use of SSCs to restore fertility. It is known that retinoids are crucial for spermatogenesis. Their importance is most clearly seen in their deficiency, which results in male infertility. Recent studies also show that retinoic acid (RA) induces meiotic entry of differentiating spermatogonia. However it is unclear if RA can modulate the activity of primitive spermatogonia. In this study we assessed the effect of RA on SSCs and primitive spermatogonia *in vitro* and used spermatogonial transplantation to measure stem cell activity. It is now possible to culture mouse SSCs and primitive spermatogonia indefinitely and expand the SSC population *in vitro*. In this culture system, SSCs form aggregates of primitive spermatogonia, called 'clusters'. Using this system, we assessed the effect of RA on clusters. When treated with RA (0.7 and 0.07 μM) for five consecutive days, clusters appeared to disintegrate and change their morphology. The cells of the disintegrating clusters formed chains of cells connected by cytoplasmic bridges, which is the morphology that has long been attributed to differentiating spermatogonia. The RA treatment increased the length of cell chains in a time-dependent manner up to 5 days, but no dose-dependent effects were observed. Using immunocytochemistry, we detected in all cluster cells the expression of retinoic acid receptor α, the major isoform of the RA receptor in mouse testes. These results suggest that cluster cells are capable of responding to RA and undergoing the morphological changes observed. To determine the effect of RA on SSCs, we quantified SSCs by transplanting cluster cells into recipient testes 2 days after the RA treatment at 0.7 μM. We detected a significant decrease in SSCs (21-fold), suggesting that RA induced SSC differentiation or death. Since all cells in clusters are known to be undifferentiated spermatogonia, application of RA into this culture system may present an effective approach to understanding the mechanism that underlying the loss of stem cell potential in the male germ line.

Poster Board Number: 2160

IDENTIFYING GENES DIFFERENTIALLY EXPRESSED BETWEEN WNT/β-CATENIN SIGNALING AND NON-SIGNALING CELLS IN MOUSE SPERMATOGONIAL STEM CELL CULTURE

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Spermatogonial stem cells (SSCs) are an elusive population in the testis but are critical for the life-long production of sperm. Recently, we investigated the involvement of canonical (Wnt/βCat) signaling in SSC fate. In a long-term culture, SSCs form aggregates (clusters) of SSCs and differentiated cells. Using a transgenic reporter mouse-line for Wnt/βCat pathway activation, we found that Wnt/βCat signaling is activated in a subpopulation of cluster cells and that Wnt/βCat-signaling cells do not have stem cell activity suggesting that they may be early committed progenitors. Therefore,

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in this study we examined the downstream targets differentially regulated in these non-SSCs. Using a global gene expression approach we identified 137 targets upregulated at least 2-fold in Wnt/βCat signaling cluster cells. Gene ontology analyses classified most targets as components of extracellular matrix and in cell adhesion. We noted the expression of members of the Notch pathway (Dlk1, Notch4, Maml2), which has known importance in the cell-cell communication necessary for embryo patterning, in particular in establishing the SSC niche in the developing *Drosophila* gonad. Therefore, as a preliminary approach we added recombinant DLK1 to clusters and assessed its effect on SSC activity. We observed that the addition of DLK1 significantly reduced SSC numbers without a corresponding decrease in total cluster cell numbers, implying that DLK1 may support differentiation. These results raise the possibility that differentiated germ cells may contribute to an SSC niche and influence SSC fate.

Poster Board Number: 2162

LIN28A REGULATES GERM CELL POOL SIZE AND FERTILITY

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The RNA-binding protein Lin28a, which regulates the biogenesis of the let-7 family of tumor-suppressor microRNAs, has been implicated in ES cell pluripotency, somatic cell reprogramming, and tumorigenesis. Lin28a is expressed in primordial germ cells (PGCs) of the developing embryo and undifferentiated spermatogonia of the postnatal testes, and is over-expressed in virtually all germ cell tumors. Previously we defined a molecular pathway whereby Lin28a influences PGC development via the let-7 target Blimp-1, a key regulator of PGC specification (West et al., Nature 2009). To further elucidate the role of Lin28a in the germ lineage, we evaluated Lin28a knockout mice. By flow cytometry and immunostaining we found markedly fewer SSEA-1+ PGCs in the genital ridges of 13.5 dpc Lin28a-null embryos compared to their littermate controls (4.8 +/- 0.5 % vs 7.3 +/- 0.6 %). Neonatal mouse testes and ovaries showed correspondingly fewer gametes, as defined by staining with the mouse vasa homologue Mvh, and adult Lin28a knockout males had reduced fertility. These data indicate that Lin28a regulates germ cell pool size from an early stage *in vivo*, and compromises fertility while not abrogating germ cell function. In addition, we have developed mouse strains with conditional over-expression of Lin28a, and are currently testing how Lin28a hyper-function influences PGC pool size, fertility, and tumorigenesis.

Poster Board Number: 2164

STEM CELLS IN ASEXUALLY REPRODUCING INVERTEBRATES, EMBRYONIC STEM AND GERMLINE CELLS SHARE COMMON, EVOLUTIONARY CONSERVED FEATURES

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In asexually reproducing invertebrates no early segregation of the germ cell lineage is observed; the lineage of pluripotent (traditionally referred to as totipotent) stem cells ensure both sexual and asexual reproduction over the entire life span of an individual or a colony. These pluripotent stem cells can differentiate into gametes and all the types (or a wide spectrum) of somatic cells in adult organisms. We studied such stem cells in representatives of five animals: archaeocytes in sponge Oscarella malakhovi (Porifera), interstitial cells in colonial hydroids Obelia longissima and Ectopleura crocea (Cnidaria), neoblasts in planarian Girardia tigrina (Platyhelmintes), stem cells in colonial rhizocephalans Peltogasterella gracilis and Polyascus polygenea (Arthropoda), and colonial ascidian Botryllus tuberatus (Chordata), compared with mouse embryonic stem cells (mESCs) *in vitro*. The morphological structure of studied stem cells in such diverse taxa shares common features typical for germline cells in other studied Metazoa: a high nucleo/cytoplasmic ratio, a large nucleus with diffuse chromatin and a large nucleolus, thin rim of undifferentiated basophilic cytoplasm including electron-dense perinuclear granules (nuage). All studied stem cells contain the specific electron-dense germinal granules or nuage - ultrastructural marker and key organelles of metazoan germline cells as well as of potentially gametogenic pluripotent stem cells. Asexually reproducing animals express evolutionary conservative germ cell markers such as products of genes related nanos, vasa and pl-10 (DEAD box family members), Piwi, and also high activity of alkaline phosphatase, PCNA and telomerase some of which were found in studied species: PCNA, alkaline phosphatase activity, selective expression vasa-like gene. We found expression of Ddx4/Vasa, Miwi/Piwi and Nanog/Nanos in undifferentiated mouse embryonic and induced pluripotent stem cells, with localization of DDX4 protein in perinuclear granules (nuage) of mESCs, which, along with invertebrate stem cells, are potentially gametogenic cells retaining pluripotency. We hypothesize that evolutionary conserved mechanism ensures germline specification remaining cell pluripotency. The data supported our hypothesis that pluripotent, potentially gametogenic stem cells display evolutionarily conservative features of the morphological and functional organization typical for cells of the germ line. In asexually reproducing invertebrates, from sponges and hydroids to some arthropods and chordates, stem cells share with cells of early embryos evolutionary conservative features presumably involved in maintenance of pluripotency (totipotency) and the gametogenic program. Such invertebrate cells capable of both gametogenesis and asexual reproduction (blastogenesis) are similar in their potential to mammalian embryonic stem cells. We propose that evolutionary and ontogenetically related cells of early embryos and pluripotent stem cells belong to populations of cells that retain a wide or unlimited morphogenetic potential.

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EXPRESSION OF POSTMEIOTIC MARKERS DURING DIFFERENTIATION OF NONHUMAN PRIMATE ES CELLS *IN VITRO*

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Primate embryonic stem (ES) cells can differentiate into pre-meiotic germ cells spontaneously *in vitro*. It has been reported that although expressions of several meiotic and post-meiotic marker genes become detectable during embryoid body (EB) differentiation from ES cells, the meiotic progression is inefficient for producing functional gametes. However, expression of post-meiotic markers such as TEKT1 and GDF9, specific for spermatid and oocyte respectively, has been reported regardless of sex difference (XY or XX) of primate ES cells. To understand the expression of these post-meiotic markers in comparison with other germ cell markers further, we investigated sequential expression of stage-specific germ cell markers during *in vitro* differentiation using XY and XX ES cell lines established from cynomolgus monkey blastocysts. Immunohistochemical analysis was also performed to identify cells expressing these post-meiotic markers in EBs. Several pre-meiotic germ cell markers (VASA, NANOS3, CXCR4) were upregulated upon the *in vitro*

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differentiation but meiotic markers were not. On the other hand, post-meiotic markers (ACROSIN, HAPRIN, TEKT1, and GDF9) were upregulated in both XY and XX cell lines. Immunohistochemical analyses of EBs showed the existence of cells expressing TEKT1 and GDF9 protein, and co-expression of both proteins was observed in some cells. In addition, some TEKT1 positive cells co-expressed Vasa, possibly suggesting the existence of germ cell lineage expressing post-meiotic genes without meiosis. Of interest, XY cells and XX ES cells showed similar time course of germ cell marker genes and appeared to be similar in the post-meiotic gene and protein expression profiles. These results suggested that sex difference (XY or XX) did not affect the expression pattern of germ cell marker genes especially TEKT1 and GDF9 under *in vitro* differentiation.

Poster Board Number: 2168

GENE EXPRESSIONAL AND EPIGENETIC SIGNATURES TO DEFINE SPERMATOGENIC CELLS OF CALLITHRIX JACCHUS

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Germ cell development is a fundamental process to produce offspring and sustain the species. As a result of evolutional selection, the developmental program of spermatogenesis seems to be quite similar across mammalian species. However, it becomes revealed that the program is not identical in detail among species. This fact prevents a simple application of rodent insights to higher primates, despite accumulated knowledge on the molecular properties of germ cells in mouse. Thus, considering an extrapolation to human, it is essential to investigate the nature of primate germ cells using appropriate model animals. Here, we performed a molecular dissection of spermatogenic cells in common marmoset Callithrix jacchus. RT-PCR and immunostaining analyses demonstrated many similarities in gene expression between mouse and marmoset testes as expected. On the other hand, we found the exclusive signatures to distinguish both species as shown by the expression of pluripotency and early germ cell markers. It was also revealed that NANOS family and PLZF proteins changed their intracellular localization during spermatogenesis, unlike mouse homologues. Furthermore, DNA methylation analyses identified a unique epigenome profile that the marmoset NANOG and OCT4 promoters exhibited an opposite methylation status to those in mouse spermatogenic cells, while the VASA and LEFTY1 loci as well as imprinting genes displayed an evolutionarily conserved DNA methylation status. Because marmoset holds great advantages as not only a human model but also an experimental animal, the current study could be an important platform for primate reproductive biology including a possible application to human.

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IDENTIFICATION OF SPERMATOGONIAL STEM CELLS ISOLATED FROM TESTIS OF OCT4-EGFP TRANSGENIC PIGS

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Spermatogonial stem cells (SSCs) are essential for initiation and maintenance of spermatogenesis in the testis of mammalian species. SSCs are unique because of their capacity for self-renewal and/or the potential for differentiation into spermatozoa. SSCs are rare in the testis and show only limited proliferation *in vitro*, hampering in-depth studies on their developmental potential *in vivo* and *in vitro*. Up to now, only mouse SSCs have been successfully cultured *in vitro* over prolonged periods of time. However, domestic animals, in particular the domestic pig, are excellent large animal models, in which the clinical potential of stem cell therapies can be studied. We had previously generated and extensively characterized Oct4-EGFP (OG2) transgenic pigs as a novel large animal model for studying the fate of pluripotent cells, by monitoring of Oct4 expression using EGFP fluorescence. SSCs are the only adult stem cells that express Oct4; thus expression of the OG2 transgene can be used to isolate SSCs in OG2 transgenic pigs. In the present study, testes were obtained from OG2 transgenic boars at different ages. For derivation of SSCs the testes were enzymatically dissociated using two digestion steps to obtain a single-cell suspension. Fluorescence-activated cell sorting (FACS) based on Oct4-EGFP and c-KIT expression was used to purify the spermatogonial stem cell population. Four cell populations, i.e. EGFP+/c-KIT-, EGFP+/c-KIT+, EGFP-/c-KIT+ and EGFP+/c-KIT+ could be isolated. Fluorescence microscopy of testicular tissue isolated from transgenic piglets revealed a very low number of EGFP positive cells, whereas testicular tissue isolated from adult transgenic boars resulted in a high amount of EGFP fluorescent cells. FACS analysis of ejaculated spermatozoa from mature OG2 boars displayed no EGFP fluorescence. At present, we are working on the further characterization of these cell populations using real-time PCR analysis and immunohistochemistry. OG2 transgenic pigs used for isolation testicular cells based on both expression of Oct4-EGFP and specific surface markers could allow improved characterization of SSCs in pigs.

TECHNOLOGIES FOR STEM CELL RESEARCH

Poster Board Number: 2172

A NOVEL PLATFORM UTILIZING FLOW CYTOMETRY ACTIVATED CELL SORTING AND DEFINED CULTURE SYSTEMS TO ENABLE THE GENERATION OF CLONALLY DERIVED HIPSCS WITH NAïVE STATUS

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Several key obstacles impede the translation of human induced pluripotent stem cells (hiPSCs) to clinical applications: the lack of an efficient feeder-free reprogramming method, a robust clonal selection strategy and a scalable single-cell culture technique. To this end we have developed a chemical platform comprising a unique combination of four small molecule inhibitors of signaling pathways associated with the perturbation of stem cell self-renewal and survival: ROCK, TGFβ, MEK and GSK3. This small molecule cocktail is seen to not only augment somatic cell reprogramming but also supports the viability, proliferation and self-renewal of established hiPSCs in a defined media formulation. Using this platform, we have demonstrated enhanced and routine feeder-free reprogramming of various somatic cells using three factors (Oct4/Klf4/Sox2) and derived the previously unattainable feeder-free hiPSCs from IMR90 fibroblast cells. In addition, we have replaced the cumbersome and often impure technique of picking potential hiPSC colonies based on morphology with an effective flow cytometry sorting and expansion strategy: capturing individual hiPSCs by unique pluripotency surface markers to efficiently obtain, expand and characterize clonal hiPSC lines. Importantly, hiPSCs derived and maintained using this platform retain genomic stability over multiple passages, effectively differentiate into the three germlayers and are seen to have minimal requirements for extrinsic signaling. Furthermore, we have demonstrated that hiPSCs previously generated in conventional feeder-systems can be adapted to our feeder-free and single cell culture platform. hiPSCs adapted to or generated in our platform are more similar to human embryonic stem cells, indicated by global expression profiling, reduced Xist activity and compact and dome-like morphol-

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ogy when reintroduced on to feeder cells; all suggestive of naïve status. This study provides a chemical platform that not only facilitates the single cell and the defined culture of clonally derived feeder-free hiPSCs but also makes possible the generation of pharmaceutical-grade hiPSCs for regenerative medicine applications.

Poster Board Number: 2174

SCALABLE EXPANSION OF UNDIFFERENTIATED HUMAN EMBRYONIC STEM CELLS IN SERUM-FREE AND DEFINED SUSPENSION CULTURE

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We have extensive experience in cGMP banking of various cell lines in support of human clinical trials, including various viral vector producer lines, human feeder lines, and transformed neural stem cell (NSC) lines. Over the past several years we have focused our attention on the development of cGMP processes for banking and differentiation of human embryonic stem cells (hESCs) in support of planned and anticipated clinical studies. We have developed an adherent hESC line suspension culture adaptation process and a scalable suspension culture system using defined reagents. We have demonstrated that this system will support extensive passaging and expansion of several hESC lines. To develop the process, we assessed different serum-free, defined media and a broad spectrum of additive factors, including kinase inhibitors, cytokines, and adhesion modulators in small-scale rotary culture. Promising conditions were further tested in small-scale spinner culture. Our data shows that hESC cultured in the presence of a ROCK inhibitor and increased bFGF supported undifferentiated growth of hESC lines as small aggregates in suspension. Ultra-structural staining, immunohistochemistry, and confocal microscopic analyses of these cellular aggregates reveal high expression of pluripotent markers on the cells comprising the aggregates. Suspension culture parameters were further improved by assessing changes in agitation rate, seeding density, and passage interval to optimize cell growth while minimizing differentiation rates. Under optimized culture conditions, we demonstrated that hESCs can be serially passaged for more than 15 times in spinner flasks and expanded in large-scale to over 1x107 fold increase. The expanded cells retained a normal karyotype, low differentiation as measured by SSEA-4, Tra-1-60 and Tra-1-81, and Oct-4, high viability (>85%), and EB forming capability. Epigenetic analysis of the suspension culture showed high similarity to fingerprints from hESC cultured on mouse embryonic fibroblasts (MEFs). The cells can be cryopreserved in serum-free medium and thawed into adherent or suspension cultures with high viability (>85%). We have successfully used this method under cGMP conditions to generate cell banks of 200-400 vials of the hESC lines HES-2, H1, and H9. Taken together, our suspension culture system provides a powerful approach for scale-up expansion of hESCs and is suitable for process transfer to our GMP facility for banking and bulk differentiation of hESC lines in support of clinical studies as a national resource.

Poster Board Number: 2176

DEFINED, XENOBIOTIC-FREE MICROCARRIER CULTURE FOR ISOLATION OF NOVEL HUMAN INDUCED PLURIPOTENT STEM CELLS AND FOR MASS PRODUCTION

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Current methods for creation of new human induced pluripotent stem cell lines (iPSC) require frequent, labor intensive culturing steps to achieve isolation of iPSC from the population of parental somatic cells that were not induced to pluripotency, and to expand the iPSC population before beginning differentiation. Two recent publications have reported the use of microcarrier based stirred-suspension bioreactors for cultivation of human embryonic stem cells (hESC) and iPSC. Both studies employed microcarriers coated with extracellular matrix extract derived from tumors grown in mice (BD Matrigel™). However, to fulfill the promise of iPSC as a clinical-grade source for regenerative and cellular therapies, xenobiotic and undefined materials (serum, Matrigel, conditioned media) must be excluded at all stages of culture, from the creation of the iPSCs through clone selection and propagation/expansion. It also is essential for consistency to automate as much of the culture process as possible, while maintaining the pluripotency and robust proliferation of the iPS cells. Primorigen is addressing these needs by developing a xenobiotic-free, feeder cell-free, scalable stirred suspension bioreactor culture system for mass production of iPSC lines using Primorigen's proprietary stem cell attachment factor, StemAdhere XF™. StemAdhere XF is a highly purified, xenobiotic-free recombinant human protein that enables calcium-dependent adhesion and proliferation of pluripotent stem cells. StemAdhere XF has been immobilized on a variety of porous and solid microcarriers, and used to expand existing iPSCs in stirred cell bioreactors.

Poster Board Number: 2178

IN VIVO AND *IN VITRO* GENE INDUCTION IN T LINEAGE CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS

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Stem cell therapies which rely on transplantation of a permanently modified population of hematopoietic cells which express a gene of therapeutic interest lack both the safety and the increased efficacy afforded by a more directly regulated expression system. To address this problem, we have modified hESC lines such that a gene of interest can be tightly regulated by treatment with doxycyclin. We derived hematopoietic precursors from these lines, and have generated thymocytes *in vivo* from these precursors using our recently reported SCID-hu based method. We subsequently elicited expression of a gene of interest *in vitro* in these cells following co-stimulation, and have further shown that an induced external reporter protein correlates directly with the level of expression of a protein of interest. We can now explore *in vivo* gene induction using these doxycylin responsive hESC derived T lineage cells, and we will present preliminary data demonstrating *in vivo* induction in the SCID-hu system. We anticipate that tight regulation of expression of therapeutically important proteins will be essential to long term patient treatment success and safety, and we thus present here one method to achieve the desirable level of control.

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Poster Board Number: 2180

HUMAN PERIPHERAL BLOOD-BORN HEMATOSPHERE (BBHS) AS A NICHE FOR LIN(-) CD34(+)CD38(-) HEMATOPOIETIC STEM CELL EXPANSION

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BACKGROUND: Human peripheral blood is a promising source of autologous stem-progenitor cells such as endothelial progenitor cells or hematopoietic stem cells However, the rarity of stem cell population and lack of effective method to expand or differentiate it remained a major hurdle for its application. Recently, Spheroid cultures of various stem-progenitor cells have been successfully used to expand or differentiate stem cells in embryonic, cardiac, neurogenic and cancer stem cells. METHODS AND RESULTS: Here, we described a novel method of Lin(-)CD34(+)CD38(-) hematopoietic stem and progenitor cells(HSPCs) expansion using spheroid culture of human peripheral blood mononuclear cells (hPBMCs) without synthetic growth factors or artificial niche. The spheres, termed as 'Blood-Born Hematospheres (BBHS). We found that self-renewal of CD34(+) hematopoietic stem-progenitor cells(HSPCs) were localized inside BBHS. Flow cytometry analysis revealed that HSPCs exponentially increased between day 3 and day 7 during BBHS maturation and these HSPCs were functional *in vitro*, colony forming assay and *in vivo*, long-term SCID repopulating cell (SRC) assay. Freshly isolated MNCs contained scanty amount of Lin(-)CD34(+)CD38(-) cells. However, at day 5 of BBHS culture, 4-6% of Lin(-) cells were CD34(+) CD38(-). The increase of absolute Lin(-)CD34(+)CD38(-) count at day 3 and day 5 were 5.2- and 9.4-fold, respectively. Culture of 2.0 x 107 MNCs, which usually corresponds to 10ml of peripheral blood from healthy donor, yielded 1.5 ± 0.2 x 105 Lin(-)CD34(+)CD38(-) cells at day 5 which corresponds to 1.6% of total cell and 7.2% of lineage-negative fraction. BBHS culture expands phenotypic Lin(-)CD34(+)CD38(-) cells average 10-fold and yields 6-fold expansion of colony forming progenitors. BBHS consists of heterogenous blood cells and CD14(+) cells were found to be the cellular component of BBHS and its exclusion disrupted the sphere formation. Furthermore, BBHS provided natural extracellular matrix deposition such as Fibronectin, laminin and collagen I,III, and IV for human stem-progenitor cells self-renewal. CONCLUSION: Here, we described a new method that high density suspension culture of peripheral blood mononuclear cells results in spontaneous formation of hematopoietic cell spheroids. The culture system, which we termed BBHS, potentiates the expansion of rare circulating Lin(-) CD34(+)CD38(-) HSPCs population in the sphere. The expanded HSPCs remained functional *in vitro* and *in vivo*. Our novel strategy of *ex vivo* expansion of human stem-progenitor cells using self-organized hematopoietic sphere provides an innovative autologous HSPCs source and a new model to investigate adult stem cell niche.

Poster Board Number: 2182

QUANTITATIVE CYTOLOGICAL FEATURE ANALYSIS BY MICROFLUIDIC IMAGE CYTOMETRY REVEALS PHENOTYPIC DIFFERENCES AMONG HUMAN PLURIPOTENT STEM CELL LINES

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Human pluripotent stem cells (hPSCs), including embryonic stem (ES) and induced pluripotent stem (iPS) cells hold a great potential for applications in cell-based therapy, drug screening and regenerative medicine as well as developmental biology due to their unique characteristics, such as (i) unlimited self-renewal at undifferentiated stage with normal karyotype and (ii) the ability to differentiate any cell types in a human body. However, recently, even though hiPSCs were generated to mimic hESC characteristics, there have been some studies reported that assess the similarities and differences between hiPSCs and hESCs. Indeed, some of the hiPSC lines appear to vary morphologically and behave differently in chemically defined media due to variables involved in the derivation/culture methods. Moreover, there are the additional concerns that hPSCs generate highly heterogeneous populations during culture and might affect the purity of targeted cells during induced differentiation. Therefore, it is necessary to develop new methodology to systematically assess the difference individual hPSC characteristics to address these issues. Microfluidic image cytometry (MIC) is advantageous over flow cytometry for profiling cell behavior at a single cell level due to its capabilities of (i) well-controlled cellular environments, (ii) higher-throughput and multiparametric assays for individual cells and (iii) *in situ* real-time cell monitoring. In these years, we have developed MIC platforms for characterizing individual hPSCs cultured in chemically defined medium and diagnosing glioblastoma patients *in vitro*. While genetic and epigenetic approaches can provide valuable information, in this study we have used MIC technique to indentify an alternative way to compare and distinguish the current state of a particular iPSC line from another iPSC line or other ESC lines and determine relative status of pluripotency by analyzing the unique cytological features of hiPSCs at a single cell level. To begin with, we established the two sets of phenotypic markers that could characterize hPSCs based on cytological features collected at single cell level. General cytological features evaluated in the experiments include, but not limited to, the size, texture, shape and maker intensity of nucleus, cytoplasm or both and the distance between neighbor cells. In terms of phenotypic markers, the first set contains 5 markers aimed to check for cell cycling and apoptosis: i) DAPI for DNA contents, the size of nucleus and G1 and G2 phase in cell cycle, ii) phosphorylated-Histone H3 and iii) EdU for M and S phases in cell cycle respectively, and iv) Caspase-3/7 for apoptosis (Phenotype assay I). The second set includes 3 markers: i) DAPI for DNA contents, ii) OCT4 and iii) SSEA1 for pluripotency and differentiation, respectively (Phenotype assay II). Finally, we have established the multi-parametric microfluidic phenotypic assays combined with biostatistic analyses (i.e., common factor analysis, self-organizing map analysis and hierarchical clustering) that provide a collection of cytological features. Interestingly, unsupervised hierarchical clustering of SOM data revealed that hESC lines and hiPSC lines were distinguished, and hiPSC lines was categorized into a same group with IMR90 somatic fibroblast cell line.

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MICROFLUIDIC CLONAL CULTURE OF HUMAN EMBRYONIC STEM CELLS

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The analysis of human embryonic stem cells (hESCs) at the single cell level is limited by low hESC plating efficiencies and limitations to analytical throughput. We have developed a sub-line of CA1 hESCs (CA1S) that can be passaged as suspensions of single cells with high plating efficiency (24 ± 4% compared to 0.4 ± 0.1% for CA1 cells). The CA1S cells display normal karyotype, growth rates, pluripotency marker expression, differentiation potential and teratoma formation. For the purpose of analyzing clonal CA1S cell cultures, we have fabricated a microfluidic cell culture platform consisting of 160 individually addressable parallel 500 x 500 um culture chambers adapted to perfusion culture of isolated hESC colonies, each in 25 nL. This device and the CA1S cell line extend the state of the art for hESC culture by analyzing colony growth from single-cell suspensions, precise quantification of cell growth by counting individual cells and observation of colony surface marker heterogeneity, during clonal cell maintenance or differentiation. The overall microfluidic device culture performance was optimized until growth rates in the chip (td = 23.4 ± 1.6 h, n=3) matched conventional hESC culture growth rates (td = 22.5 ± 2.9 h, n=3) and exhibited normal colony morphologies. Microfluidic immuno-staining showed that the cells cultured on chip remained positive for Oct4, Tra-1-60 and SSEA-3. Furthermore, cells recovered from the device were positive for cell surface markers associated with the undifferentiated state such as SSEA-3 (99.8% positive) and TRA-1-60 (96.5% positive). This system provides live-cell imaging of many isolated conditions for dynamic medium change investigations and for directed differentiation of hESCs, resolving cell-to-cell and colony-to-colony variations.

Poster Board Number: 2186

COMPARATIVE ANALYSIS OF GENE EXPRESSION AND FIBROBLAST COLONY FORMING ABILITIES OF HUMAN ADIPOSE OR UMBILICAL CORD DERIVED PROGENITOR CELLS

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Mesenchymal stem cells (MSCs) from adipose tissue (AT) or umbilical cord (UC) are regarded to be important for the source of regenerative medicine. Recent studies have suggested stable expansion of cell growth and maintenance of intrinsic characteristics are necessary to investigate or develops a cell therapy. Therefore, this study investigates the gene expression profiling and the frequencies of colony-forming unit-fibroblast (CFU-F) of human AT- or UC-MSCs. The cells were prepared by enzymatic dissociation and characterized by flow cytometry (CD31, CD34, CD90, CD45, and CD117). Gene expression was analyzed by NimbleGen Human Whole Genome 12-plex Array and hybridization by MAUI system. Functional genes related signal transduction, transport, differentiation and immune response showed differential expression after *ex vivo* expansion. Of 44,049 genes, 917 genes significantly expressed in AT-MSCs compared to UC-MSCs. Especially, gene expression of inflammatory response, cell migration, growth and metastasis showed similar patterns (< 8 genes). After a culture period of 14 days, proliferation potential of CFU-F was evaluated. Human umbilical vein endothelial cells (HUVECs) were used as negative control and had a disability of colony-forming. By image analysis, results of the UC-MSCs increased more than 55 percent the rate of AT-MSCs. Population doubling levels of both MSCs showed a high rate (> 35th doublings), although their value of CFU-F was indicated significant difference. These data indicate that AT- or UC-MSCs have similar gene expression profiling and high proliferation ability. Further studies are required to investigate the characteristics and differentiation ability of AT- or UC-MSCs in modified conditions or long-term culture.

Poster Board Number: 2188

REVERSAL OF TYPE 1 DIABETES THROUGH THE IMMUNE MODULATION OF HUMAN CORD BLOOD-DERIVED MULTIPOTENT STEM CELLS

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Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease that results in a shortage of pancreatic islet β cells. To date, many approaches have been investigated to find a cure for T1D. All in all, control of autoimmunity is the top priority for the prevention and treatment of T1D, followed by the provision of β-cell surrogates and reconstruction of functional islets. If unaddressed, the autoimmune cells can destroy those β-cell surrogates and limit their therapeutic potential. Due to the polyclonal nature of the autoimmune response and the global problem of immune regulation in T1D, approaches that individually targeting different arms of the immune system have been uniformly unsuccessful in T1D prevention trials. Stem cells hold great promise for the treatment of T1D in overcoming the shortage of insulin-producing cells. For a long time, most work on stem cells has focused on regulating their differentiation to replace a specific physiologic function. We identified a novel type of stem cell from human umbilical cord blood, designated cord blood-derived multipotent stem cells (CB-SC), which display embryonic stem (ES) cell characteristics and multiple potential of differentiations. Recently, our animal data revealed that CB-SC can function as an immune modulator that can lead to control of autoimmune responses, which could in turn be used as a new approach to overcome the autoimmunity of T1D in patients. *In vitro* co-culture experiments demonstrated that direct suppression of CB-SC on the islet β-cell GAD-specific CD4+ T cell clone generated from T1D patients. The proliferation of this T cell clone was markedly decreased in the presence of CB-SC compared to the control group in the absence of CB-SC. To translate this technology into clinics, we invented a device Stem Cell Educator that is optimized for CB-SC culture and clinical treatment via connecting with Blood Cell Separator. In collaboration with a medical center in China, Phase I clinical trial (n = 19, 10 males, 9 females) has demonstrated the feasibility and safety of Stem Cell Educator therapy. Ongoing studies have confirmed that T1D patients (n = 9, aged 15-48 years, diseased for 1-17 years) receiving Educator therapy experienced improvement of metabolic control, a marked reduction of HbA1c values, decreasing insulin doses, and increasing C-peptide values both at baseline and 2 hours post high glucose challenge. Additionally, immune characterizations have revealed the reversal of autoimmunity of T1D, the restoration of Th1/Th2 cytokine balance, the marked up-regulation of Foxp3+ regulatory T cells (Tregs) in peripheral blood. The whole procedure is simple, safe and very cost-effective. The Autoimmune Regulator (Aire) usually expressed in thymic medullary epithelial cells, plays an important role in immune tolerance by mediating the ectopic expression of peripheral self-antigens and the deletion of auto-reactive T cells. To explore molecular mechanisms underlying the immune modulation of CB-SC, we found CB-SC express Aire at both mRNA and protein levels. Using three pairs of human Aire-specific small interfering RNAs (siRNA) to knockdown Aire expression, functional analysis demonstrated that expression of Aire in CB-SC contributes to the immune modulation. Thus, our findings have the potential for enormous clinical

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impact on T1D, by paving the way toward the development of novel Stem Cell Educator therapy to reverse diabetes in T1D patients.

Poster Board Number: 2190

CXCR1 MEDIATED MIGRATORY CAPACITY OF HUMAN UMBILICAL CORD BLOOD-DERIVED MESENCHYMAL STEM CELLS TOWARD GLIOMAS

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The use of tumor-tropic mesenchymal stem cells (MSCs) as a delivery vehicle of antitumor agents is a promising approach in cancer gene therapy. However, achieving a sufficient number of tumor-targeted MSC may be a limitation of this strategy. Interleukin-8 (IL-8) released by glioma cells mediate the tropism of MSC toward tumors. Here, we show that transfection of the IL-8 receptor, CXCR1, on human umbilical cord blood-derived MSCs (UCB-MSCs) enhanced the migratory capacity of MSC toward gliomas. We showed that UCB-MSCs have the migratory ability toward the glioma cell lines and primary glioma cells. IL-8 treatment increased the migration of UCB-MSC in a dose-dependent manner and inhibition of IL-8 by treatment with the anti-IL-8 or IL-8 siRNA transfection into glioma cells blocked the migration capacity of UCB-MSC toward glioma cells. Furthermore, CXCR1-transfected UCB-MSCs (CXCR1-MSCs) showed a superior migratory capacity toward glioma cells compared with primary MSCs *in vitro* migration experiment, and also exhibited enhanced migration toward tumors in mice bearing intracranial human glioma by the histological and *in vivo* imaging analysis. Our findings indicate that overexpression of CXCR1 may be a useful tool for MSC-based gene therapy to achieve desirable sufficient quantity of therapeutic MSC that localize within tumors.

Poster Board Number: 2192

GENERATION OF ICM-TYPE HUMAN IPS CELL FROM CD34+ CORD BLOOD CELLS

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Mouse ES cell lines from C57BL/6N or 129Sv strain are the ICM-type stem cells. They can contribute to chimerism when placed into blastocyst and be passaged in a single cell suspension. However human ES cells or iPS cells cannot contribute to chimerism and retain the similar gene expression pattern to the mouse Epiblast. They are called the Epistem-type stem cells and can be passaged as cell clumps. In this study, we have reprogrammed CD34+ cord blood cells with Sendai virus integrating factors (Oct3/4, Sox2, c-myc, Klf4) and by culturing the infected cells in the ICM-type stem cell culture condition [N2B27 based media supplemented with LIF, GSK3 inhibitor, MEK inhibitor and forskolin under 5% O2 hypoxic condition] for single cell cloning. Approximately five dome shape-like, colonies emerged from 1X104 freshly isolated CD34+ cord blood cells around 14 days when co-cultured with feeder cell line SNL. Removal of SeV construct from the reprogrammed cells was examined in single cell level by RT-PCR and immune-staining for viral construct during several passages in the ICM-type stem cell culture. As we cannot maintain the ICM-type stem cell by this culture method for more than 10 passages, reprogrammed ICM-type stem cells were converted to EpiSCs-type by changing culture condition to KSR-based human ES media with bFGF in 20 % O2 condition for further appraisal of differentiation potential of reprogrammed cells. Our report would provide the safer iPS generation method for future clinical application of iPS cell-derived cell therapy.

Poster Board Number: 2194

THE ROCK INHIBITOR Y-27632 PROMOTES MOTOR NEURON GENESIS, MOTOR NEURON SURVIVAL AND NEURITE OUTGROWTH IN CULTURES DERIVED FROM HUMAN EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS

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Human Embryonic Stem Cells (hESCs) and Induced Pluripotent Stem Cells (iPSC) can virtually give rise to all cell types in the body, constituting an inexhaustible source of disease relevant cell types, which creates new exciting opportunities in the study of incurable neurodegenerative disorders like Amyotrophic Lateral Sclerosis (ALS), where affected cell types are naturally hard to obtain. Conditions for patterning of hESC and iPSC cells resulting in specification of motor neurons (MNs) have been extensively studied and in general work robustly. However, less is known about the mechanisms that control motor neuron genesis and human motor neuron survival. Here, using human ES-derived MNs, we performed a medium-throughput screen to identify compounds that increase MN numbers and found the putative Rho-associated Kinase (ROCK) Inhibitor Y-27632. We demonstrate that this molecule increases MN numbers approximately 5-fold over 09 days treatment comparatively to control conditions. We dissected the mechanisms and demonstrate that Y-27632 compound provides a small, but significant increase in motor neuron survival and neurite outgrowth - two effects previously attributed to the Y-27632 compound in other cellular contexts. Interestingly, the most significant effect was on increased cell proliferation leading to the amplification of the number of progenitor cells in MN lineage and human motor neurons. This was also documented in MN cultures derived from several wild-type hESC and iPSC cell lines. In conclusion, we identified a reagent that increases motor neuron yields and will facilitate more efficient utilization of hESC or iPSC derived MNs for cell-based screening and other clinical applications.

Poster Board Number: 2196

DEVELOPMENT OF HIGHLY SENSITIVE ASSAYS TO DETECT RESIDUAL HUMAN EMBRYONIC STEM CELLS FROM HUMAN EMBRYONIC STEM CELL DERIVED CARDIOMYOCYTES

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Human embryonic stem cell (hESC) can be differentiated to pure populations of a variety of cell types, providing a promising source of cells for disease therapy. However, a major concern with hESC-derived cell therapies is the possibility of teratomas from residual embryonic stem cells. To address this concern we developed a functional assay, hESC-like colony formation, to detect very rare residual embryonic stem cells in embryonic stem cell-derived cardiomyocyte preparations. When undifferentiated hESCs are spiked as single cells into a lot of hESC-derived cardiomyocytes, the appearance of colonies is directly proportional to the input hESC cell number. Furthermore, we have identified the presence of very low-levels of residual cells capable of forming colonies in unpurified hESC-derived cardiomyocytes utilizing this assay. In parallel, we have developed an assay to accurately identify and

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quantitate rare OCT4A expressing cells in a mixed population. Using an immunofluorescent high content analysis approach, we are able to accurately identify spiked hESCs in a lot of highly purified hESC-derived cardiomyocytes in a linear fashion down to less than 0.0002%. We performed FACS to divide the cardiomyocyte preparation into sub-populations based on surface marker expression and discovered that the colony forming activity is restricted to cells that express a number of canonical hESC markers. Interestingly, we found that the number of colonies correlated with the levels of OCT4A expressing cells. This population of OCT4A positive cells is a very small fraction of the cells within this stem-cell like population. The remaining hESC-derived cardiomyocyte population contained no detectable OCT4A+ cells and no colony-forming ability. Here we demonstrate the ability to detect residual hESC-like cells with remarkable sensitivity using a functional colony-formation assay and a high content analytical screen to detect OCT4A expressing cells. This addresses a critical safety issue for the use of hESC-derived cells in cell transplant therapy.

Poster Board Number: 2198

ESTABLISHMENT OF GENE EXPRESSING ARTIFICIAL CHROMOSOMES IN HUMAN EMBRYONIC STEM CELLS

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Development of efficient gene delivery platforms in human embryonic stem cells (hESc) is vital to realizing their full potential for basic and clinical studies. The purpose of this study was to develop an efficient, non-integrating gene expression system in hESc using human artificial chromosomes (HAC). Similar to endogenous chromosomes, HAC are capable of gene expression, replication and segregation during mitosis. HAC present certain advantages over conventional gene delivery vectors, as they do not integrate into the host genome and can encompass large genomic regions for the delivery of multiple genes. Despite the advantages HAC offer, their use has been limited due to low cloning and transformation efficiencies, thus only studied in immortalized and tumor derived human cell lines. In this study, we utilized the high transduction efficiency of Herpes Simplex Type-1 (HSV-1) amplicons to overcome the aforementioned difficulties and subsequently delivered HAC vectors into hESc. Analysis of stable hESc clones showed that *de novo* gene- expressing HAC were present at high frequencies without integrating into the host genome. Established HAC contained an active centromere, and were stably maintained without integration or loss in the absence of selection for 30 passages. Stable HAC containing hESc clones retained their pluripotency and differentiation capabilities as demonstrated by neuronal differentiation, *in vitro* germ layer and teratoma formation assays. Our findings are proof of principle study that hold potential for delivering high capacity genomic constructs safely and efficiently into hESc for the purpose of genetic manipulation and ultimately, somatic gene therapy.

Poster Board Number: 2200

HOMOLOGOUS RECOMBINATION IN HUMAN PLURIPOTENT STEM CELLS USING HELPER-DEPENDENT ADENOVIRAL VECTORS

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Increasing the efficiency of gene targeting in human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) is essential to improve both the experimental and therapeutic potential of these cells. Although gene targeting by electroporation has been routinely used in mouse embryonic stem cells (mESCs), its application in hESCs and hiPSCs has been limited. By using high-capacity helper-dependent adenoviral vector (HDAdV), we previously reported ~45% of relative targeting efficiencies at the HPRT1 locus in hESC lines. In this study, we expanded the general applicability of HDAdVs to both gene knock-out and knock-in at both transcriptionally active (KU80, LIG1, LIG3) and inactive (motor neuron-specific HB9) loci in hiPSCs. At these five loci, 3 to 59% of vector chromosomal integration (G418-resistant colonies; G418R) were found at the target loci, judged by PCR analyses, indicating a strong affinity of the gene targeting cassettes delivered by HDAdVs with homologous chromosomal sequences. After detailed Southern analyses with multiple probes and enzymes, 68-100% of these site-specific integration events were confirmed to be mediated by accurate homologous recombination (HR) without additional ectopic integrations, resulting in overall gene targeting efficiencies of 7-81% without detectable effects on undifferentiated state and pluripotency. As one example, to examine the efficiency of HDAdV-mediated gene targeting at transcriptionally inactive loci, reporter hESC and hiPSC lines were established, in which the enhanced green fluorescent protein (EGFP) gene was knocked into the HB9 locus by HR. HB9 is expressed selectively in motor neurons in the developing vertebrate central nervous system but not in ES/iPS cells. Southern analyses demonstrated that 57% of G418R/ GANCR colonies, originated after infection with the HB9-GFP knock-in HDAdV, had been accurately targeted at the locus. The HB9 locus was also targeted in the hiPSC line at similar efficiencies, indicating that HDAdV-mediated gene targeting is equally efficient regardless of the transcriptional activities of target loci. Subsequently, the HB9-EGFP knock-in hiPSC line was induced to differentiate into motor neurons. Immunofluorescence analysis revealed a correlation between EGFP and HB9 expression, suggesting the EGFP gene was precisely knocked into the target chromosomal site by HDAdV-mediated gene targeting. HR has to be highly accurate for applications such as establishment of knock-in hESC/hiPSC lines to obtain tissue-specific expression of an inserted reporter gene. Furthermore unintended mutations during chromosomal manipulation are obviously not acceptable for therapeutic applications, such as gene repair therapy using patient-specific iPSCs. Our results indicate that HDAdVs would be the best suited for manipulation of iPSCs for clinical applications because of the highly efficient and accurate gene targeting with minimal cytotoxicity.

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Poster Board Number: 2202

STENCIL PATTERNING METHOD IMPROVES UNIFORMITY OF HUMAN PLURIPOTENT STEM CELL COLONIES

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Geometric factors including the size, shape, and density of pluripotent stem cell colonies, as well as the spacing between neighboring colonies, play a significant role in the maintenance of pluripotency and in cell fate determination. These factors are impossible to control using standard tissue culture methods. As such, there can be substantial batch-to-batch variability in cell line maintenance and differentiation yield. Not only does this variability pose a challenge in the laboratory setting, but it is a significant barrier to developing large scale cell production methods for clinical applications. We have developed a simple, robust technique for patterning Matrigel using a thin silicone stencil. We have observed that patterned arrays of Matrigel spots lead to human induced pluripotent stem cell (hiPSC) colonies which are highly uniform in growth rate, size, and shape. Patterned cell colonies are capable of undergoing directed differentiation into spontaneously beating cardiomyocyte clusters. We anticipate that this patterning method can improve both yield and repeatability in many stem cell differentiation procedures.

Poster Withdrawn

Poster Withdrawn

Poster Board Number: 2206

ATOMIC SIGNATURE OF MOUSE AND HUMAN PLURIPOTENT STEM CELLS

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The mechanisms underlying pluripotency in embryonic and reprogrammed stem cells are unclear. In this work we characterized the pluripotent towards differentiated state through analysis of trace elements distribution using the Synchrotron Radiation X-ray Fluorescence Spectroscopy. Embryoid bodies derived from embryonic and induced pluripotent stem cells were irradiated with a spatial resolution of 20 µm to make elemental maps and qualitative chemical analyses. Results show that these embryo-like aggregates exhibit self-organization at atomic level. Consistent elemental polarization pattern of S and P in both mouse and human pluripotent stem cells were observed, indicating that neural differentiation and elemental polarization are strongly correlated.

Poster Board Number: 2208

THE SCALE-UP OF HUMAN EMBRYONIC STEM CELL CULTURE USING A HOLLOW FIBRE PERFUSION BIOREACTOR - THE QUANTUM CELL EXPANSION SYSTEM

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The successful translation of allogeneic stem cell therapies to treat large patient population indications requires the realisation of economies of scale to produce a consistent final product at a price point that the healthcare sector can afford. This goal necessitates the scale-up of cell culture, which traditionally takes place in the lab setting on tissue culture plastic. However, at larger scales, such methods demand an ever-increasing 'footprint' in expensive processing facilities. Therefore, alternative culture systems characterized by high surface/volume ratios such as microcarrier or fibre based systems will likely need to be employed once the required cell number per batch rises above a critical point. Here we used the Quantum Cell Expansion system (Provided by CaridianBCT and Beckman Coulter), a hollow fibre based perfusion bioreactor system to grow a human embryonic stem cell line in a co-culture system with inactivated mouse embryonic fibroblasts. The Quantum system is a functionally closed and temperature controlled system

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comprised of ~10,000 hollow fibres of diameter ~200µm, generating a total area available for growth of 2.1m2 (21,000cm2). The machine's physical dimensions are 48cm X 50cm X 58.4cm. Cells are grown on the inside of the fibres, where they experience a low shear environment. Oxygenation is provided via a gas transfer module, through which media on the outside of the fibres is circulated. The semi-independent flow paths on the inside and outside of hollow fibers enable glucose and lactate to transfer between the two loops, while any growth factors or proteins larger than ~15kDa remain on the inside loop with the adherent cells. A karyotypically abnormal hESC line was used in the experiments, due to its relative insensitivity to its own seeding density. Consequently, fewer cells are required to achieve a seeding density capable of supporting good proliferation on the large surface area of the system. Despite its abnormalities, the line still expresses pluripotency markers (e.g., Oct 4, TRA 1-60, SSEA-4) and will form embryoid bodies, which stain positive for markers representative of the three germ layers, Sox 17, Nestin and Brachyury after re-plating. One hundred million hESC were seeded into the system atop two hundred and ten million mitomycin-C-inactivated MEFs and cultured for 5 days. Flow rates were altered to control glucose and lactate levels to within that seen in flask culture. Data will be presented from the analysis of the harvested cells, detailing the expansion and viability (Vi-CELL Trypan Blue exclusion), maintenance of pluripotency marker expression from flow cytometry and PCR, and the sustained ability to form the three germ layers after embryoid body formation.

Poster Board Number: 2210

BFGF, TGFβ1 AND PDGF SUPPORT EXPANSION OF PRIMARY HUMAN MESENCHYMAL STEM CELLS IN SERUM-FREE CULTURE MEDIUM

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As an increasing number of stem cell therapies are explored and moved into early phase clinical trials, the demand rises for animal-free, chemically-defined culture media that avoid the pathogen-safety risk and variability associated with animal serum. Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into various mesodermal lineage cell types and are currently being explored for the treatment of diseases including multiple sclerosis and graft-versus-host disease. We aim to develop an animal-free medium that supports rapid MSC expansion *in vitro*, while maintaining their stem cell phenotype and multipotency. First, MSC growth rates were monitored using the Scepter™ cell counter in serum-containing medium supplemented with bFGF. We demonstrate that glucose level, culture density and atmospheric oxygen concentration are critical parameters affecting growth rates. Stem cell phenotype was determined in early, mid and late passages by RT-PCR or immunofluorescence of MSC markers, including Stro-1, CD44, CD90 and CD105. Adipogenic and osteogenic differentiation potentials were evaluated in later passages by immunocytochemistry. Maintenance of stem cell phenotype and differentiation potential was observed beyond passage ten. Next, a basal medium formulation was developed for the expansion of MSCs in the absence of serum. This formulation was supplemented with human serum albumin (HSA) and the growth factors bFGF, TGFβ1 and PDGF, and supported higher growth rates of MSCs than did serum-containing medium. Interestingly, MSC growth varied significantly when different fractions of albumin, devoid of globulins, fatty acids or proteases, were compared. Further experimentation includes chemical composition analyses of serum fractions to identify molecules critical for MSC growth, as well as the examination of additional growth factors including BMPs and Wnt family members.

Poster Board Number: 2212

CELLFINDER: A NEW STEM CELL AND DEVELOPMENTAL BIOLOGY PLATFORM AND DATA REPOSITORY

Stachelscheid, Harald[1], Damaschun, Alexander[1], Lekschas, Fritz[1], Werner, Stefanie[1], Leser, Ulf[2], Nguyen-Dobinsky, Trong-Nghia[3], Kurtz, Andreas[1]

*[1]Berlin Brandenburg Center for Regenerative Therapies, Charité - Universitaetsmedizin Berlin, Berlin, Germany, [2]Institut für Informatik, Humboldt-Universität zu Berlin, Berlin, Germany, [3]Department of Obstetrics, Charité - Universitaetsmedizin Berlin, Berlin, Germany*Cellular therapies are increasingly relevant for personalized medicine, requiring resources informing about the complex characteristics of cells. Large amounts of research data on stem cells are already available, but these are scattered, derived by diverse technologies, not standardized and are not available at the necessary integration level for cellome modeling. Consequently, the selection of cells, e.g. for therapeutic applications, is based on rather incomplete information. CellFinder aims at mapping stem cell information to lift the cellome to an even level with the other 'omics' fields to provide a basis for global understanding of cells, increase comparability of data and relate cells to more complex systems. To organize the data stored in CellFinder we have developed a novel ontology that allows the annotation of data from the organ down to the single cell level and mapping of homologous entities between species. Furthermore this ontology allows mapping of developmental data like cell lineage differentiation. Existing taxonomies such as the gene ontology (GO) or Foundational Model of Anatomy (FMA) are used for data annotation. Based on this the CellFinder platform provides the framework for comprehensive descriptions for all human tissues, cells and commonly used model organisms on molecular and functional levels, *in vivo* and *in vitro*. These descriptions can be attributed with state-of-the-art experimental data, images, references to publications, and to sources of the relevant materials. Access to the knowledge base is provided by means of a public web interface that allows browsing, searching and querying the data stored. Current work concentrates on the integration of existing cell biology datasets to assimilate the diverse dimensions of data spanning molecular, functional, anatomical and cyto-histological, as well as auxiliary levels. The CellFinder repository will aid standardization and comparability of complex datasets for each cell and organize these in a cellome environment by ontological description and technical implementation. Future development includes community and scientific networking applications that will allow users to store and analyse their own data and to explore cells and their interactions on singular and complex resolution levels.

3D VERSUS 2D CULTURE OF HUMAN ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS (MSC)

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Mesenchymal stem cells (MSC) are multipotent stem cells, which because of their differentiation capacity and their stemness raise big hopes for therapeutic regenerative approaches. It has recently been suggested that MSC grown in spheres show higher potency, immunomodulatory and differentiation potential than MSC grown in 2D. We generated 3D MSC spheres and compared these with 2D adherently grown MSC. The differentiation capacity and stemness was analyzed by differentiation assays for neurogenesis, osteogenesis and adipogenesis. The expression of pluripotency associated genes such as Oct4, Sox 2 and Nanog as well as expression of specific differentiation markers was investigated by RT-PCR. On the protein level, we compared the proteome patterns by high-resolution 2D gel electrophoresis and nLC MALDI TOF/TOF mass spectrometry. 3D cultured MSC reacted

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faster and more effective on differentiation induction, which was proved by phenotypical staining. They also expressed higher RNA levels of differentiation markers and pluripotency markers. By high-resolution 2D gel electrophoresis 356 significant differently expressed proteins were evaluated and identified by mass spectrometry. The different *in vivo* effects of the cells are analyzed by applying the §D MSC in a Parkinson`s rat model. The results suggest a higher functional capacity of spheroid grown MSC, suggesting that the therapeutic application of MSC can be improved by spheroid or 3D cultivation methods of the cells.

Poster Board Number: 2216

DYNAMIC OPTICAL MOLECULAR IMAGING REVEALS INSIGHTS INTO HUMAN NK CELL-MEDIATED CYTOTOXICITY *IN VIVO*

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Introduction: The cytotoxic response of natural killer (NK) cells against tumor cells contributes to the beneficial clinical use of NK cells for the treatment of acute myeloid leukemia (AML). Using molecularly-specific fluorescent probes and a novel intravital fluorescence confocal microscopy (CFM) technology, our goal was to understand the spatio-temporal dynamics of NK mobility and tumor-targeting as well as the perforin-granzyme pathway of NK cell cytotoxicity *in vivo*. Our specific aim was to visualize effector-target interactions *in vitro* and extend this *in vivo* using a transparent window chamber (WC) tumor-bearing mouse model for real-time and non-invasive imaging of NK cell mobility and cytotoxicity. Methods: GFP-expressing K562 and PKH26-labled KHYG-1 cells were used in combination with an anti-perforin antibody delivered into the KHYG-1 cells using a ChariotTM vector assay. Both cell lines were co-cultured at a 10:1 effector:target ratio, and incubated at 37oC/ 5% CO2 . Aliquots of the mixed population were fixed onto microscope slides at 0, 30 min, 1, 2 and 3 h following co-culture, and imaged using an epifluorescence microscope (AxioObserver). In a separate experiment, 1Ξ106 GFP-K562 cells were injected into the fascia of female WC-bearing nude mice to induce tumor formation for one week. Then 10Ξ106 PKH26-KHYG-1 cells were injected i.v. Sytox-blueTM and CD31-APC 'reporter' probes were then injected i.v. to visualize dead cells and vasculature, respectively. Intravital confocal fluorescence microscopy (iCFM) images were obtained at 1, 2, 3, 4, 5 h and 1, 2, 3, and 4 days following injection of the reporter probes. Results: *In vitro* fluorescence microscopic imaging revealed the transfer of perforin into K562 cells from KHYG-1 (at 30 min post co-culture) with a polarized intracellular biodistribution of perforin granules. Nanotubules were observed forming between target and effector cells at 15 min post co-culture, suggesting a spatio-temporal dependency between nanotubules and perforin-mediated cytotoxicity. iCFM imaging showed KHYG-1 cells remained primarily in the vasculature up to 24 hrs after i.v. injection in K562 tumor-bearing mice. At later time points, KHYG-1 cells were observed to extravasate from the capillary network into the tumor stroma and migrate toward GFP-K562 cells. We focused on a region of interest containing a cluster of K562 cells and several KHYG-1 cells over 4 days and observed a dramatic reduction in GFP fluorescence signal in the K562 cells directly adjacent to KHYG-1 cells. Sytox-blueTM fluorescence (injected i.v.) at day 3 confirmed K562 cell death, indicating KHYG-1 mediated lysis *in vivo*. Taken together, these results demonstrate that it is possible to i) optically label effector and target cells separately, ii) track them in real-time *in vivo* through tumor vasculature while they home to the targets, iii) obtain single-cell image resolution, and iv) visualize the spatio-temporally dynamic mechanisms involved in NK cell cytotoxicty. Conclusions: While preliminary, these results demonstrate that our unique preclinical experimental intravital imaging platform offers an unprecedented ability to study the dynamic mechanism(s) of NK cell cytotoxicity in tumors *in vivo* which may help enhance our knowledge and thus the clinical effectiveness of NK cells in the treatment of AML. This new experimental platform may also have wider application in related preclinical *in vivo* studies in regenerative medicine and tissue engineering.

Poster Board Number: 2218

AN ENHANCED CHEMICALLY DEFINED SILAC CULTURE SYSTEM FOR QUANTITATIVE PROTEOMICS STUDY OF HUMAN EMBRYONIC STEM CELLS

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Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)-based quantitative proteomics analysis provides an unprecedented tool for the study of mechanisms underlying the self-renewal and differentiation of human embryonic stem (hES) cells. While we recently reported a chemically defined SILAC culture system specific for a rare cell proteomic reactor (Mol Cell Proteomics. 2010 Sep 7), total hES cell yield, prolonged self-renewal capacity (i.e. <12 days), and laborious procedure remain substantial hurdles for its conventional application in hES cell studies. Here, we devised an enhanced SILAC culture system consisting of a new chemically defined SILAC-medium and a novel culture protocol. As a result, with much less culture maneuvers, approximately 40-fold greater hES cells were produced than the system reported previously. Moreover, the enhanced SILAC culture system was sufficient to support the self-renewal of hES cells for >60 days and was also highly reproducible. As such, it provides a new platform that can be readily adapted by general laboratory for further comprehensive SILAC-based proteomics analysis of hES cells and induced pluripotent stem cells.

Poster Board Number: 2220

IDENTIFICATION OF HIGHLY SPECIFIC LECTIN BIOMARKERS FOR ISOLATION OF HUMAN PLURIPOTENT STEM CELLS

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Rapid and reliable methods for determining pluripotency in human pluripotent stem cell (hPSC) populations are critically needed, not only for quality control in basic research but also for purification of differentiated hPSCs intended for clinical use. Antibodies targeting cell surface antigens are commonly used to identify pluripotent cells in preparations of viable cells. These pluripotency-associated antigens are often glycoproteins or glycolipids, suggesting that unique glycosylation patterns potentially recognized by specific lectins may be a hallmark of pluripotency. Using protein microarrays containing 45 different lectins, we discovered unique glycosylation patterns and distinctive lectin-binding signatures that distinguish pluripotent cells from non-pluripotent cells, identified by analysis of glycoproteins extracted from 26 hPSC and 15 differentiated cell samples. As few as 15 lectins were sufficient to accurately distinguish hPSCs from non-pluripotent cell types. These highly specific biomarkers were shared by all 12 human embryonic stem cell and 14 human induced pluripotent stem cell samples examined, regardless of the method of derivation, the culture condition, the cell type of origin, and the reprogramming strategy used. We then demonstrated the

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utility of specific lectins as tools for identification and isolation of hPSCs from mixed cell populations. Lectin-mediated fluorescence staining showed extremely high concordance with staining for validated pluripotency-associated transcription factors, such as OCT4/POU5F1. In addition, we were able to separate a 1:1 mixture of hPSCs:non-pluripotent cells into two cell populations that were 90% pure using lections conjugated to magnetic beads. Gene expression analysis using cDNA microarrays suggested the differential expression of fucosyltransferases and sialyltransferases may be the mechanism underlying the association between pluripotency and specific patterns of protein glycosylation. Our results demonstrate that protein glycosylation differs considerably between pluripotent and non-pluripotent cells, and suggest that regulation of pluripotency-associated glycoproteins may be mediated by differential gene expression of glycosyltransferases. Specific lectins that recognize the pluripotency-associated glycoproteins are potentially useful biomarkers that can be used to monitor pluripotency in stem cell populations and separate pluripotent and non-pluripotent cell populations for both research and therapeutic purposes.

Poster Board Number: 2222

INSTANT REAL TIME DETECTION OF LIVE RODENT AND HUMAN NEURAL STEM AND GLIOMA-DERIVED STEM CELLS BY AN OLIGOTHIOPHENE DERIVATIVE

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The development of molecular probes for non-invasive live cell detection of specific cell types is a critical issue in cancer and stem cell biology as well as drug discovery. Here we report the synthesis of a luminescent conjugated oligothiophene (LCO), named p-HTMI, which could be used in conventional microscopy for near instant real-time detection of live embryonic neural stem cells, but not other types of physiological stem cells, differentiated cells, or cancer cells investigated. Interestingly, p-HTMI stained c:a 1-2 % of neural tumor (glioma) cells but 100% of glioma-derived stem cells, whereas the reverse pattern was seen with another LCO, pHTES, having an alternative side chain functionalization. Cell sorting experiments proved that neural and glioma-derived stem cells could be specifically detected in samples of mixed cell types. p-HTMI is functionalized with a methylated imidazole moiety resulting in a structure similar to methylated histidine/histamine and importantly a non-methylated analogue did not show the same characteristics. We propose that LCOs with distinct and defined side chain functionalities represents a novel generation of molecular probes for immediate and specific detection of specific stem cell types.

Poster Board Number: 2224

R26GR: A CRE-ACTIVABLE R26 DUAL FLUORESCENT PROTEIN REPORTER MOUSE

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Green fluorescent protein (GFP) and its derivatives are the most widely used molecular reporters for live cell imagining. Recent development of organelle-specific fusion fluorescent proteins improves the labeling resolution to a higher level. Here we generate a R26 dual fluorescent protein reporter mouse, activated by Cre-mediated DNA recombination, labeling target cells with a chromatin-specific EGFP and a plasma membrane-anchored monomeric cherry fluorescent protein (mCherry). This dual labeling allows the visualization of mitotic events, cell shapes and intracellular vesicle behaviors. We expect this reporter mouse to have a wide application in developmental biology studies as well as cancer/stem cell lineage tracing.

Poster Board Number: 2226

TECHNOLOGY THAT IMPROVES GENE TARGETING EFFICIENCY IN MURINE EMBRYONIC STEM CELLS

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Gene targeting in human embryonic stem (ES) cells and somatic cells has important applications in areas where rodent models do not adequately recapitulate human biology or disease progression. However, these applications require targeting frequencies much greater than the levels achievable with today's technology. In human ES cells and induced pluripotent cells (iPS), gene targeting is difficult and the frequencies observed are often much lower than those observed in murine ES cells. Currently, gene therapy relies heavily on viral-mediated approaches, which although successful, can also lead to serious complications. Whereas viral vectors provide efficient gene delivery, safety is a major concern, in part because random integration of the vector may cause inactivation or activation of endogenous genes leading to potentially serious side effects. Therefore, a better biological approach for gene therapy utilizes homologous recombination (HR), not only because it is inherently specific, but also because it avoids the use of viruses. Another technology which utilizes an engineered zinc-finger-endonuclease fusion protein has been shown to increase the frequency of gene targeting. However, this approach potentially can create DNA strand breaks caused by the fusion endonuclease at sites other than those intended which may lead to deleterious mutations and also create off-target nonhomologous end joining mutations. We have developed a gene-targeting technology that relies on the cell's own homologous recombination machinery. In this technology, the HR frequencies are significantly increased by using targeting vectors modified so as to contain two different peptides that, in concert, function to promote HR. The first peptide consists of a nuclear localization sequence (NLS)

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and increases the amount of tranfected DNA trafficked into the nucleus. The second peptide ("bait peptide") binds and recruits RAD51 onto the vector DNA. RAD51 (the eukaryotic analogue of the bacterial recA protein) provides enzymatic functions for recognition of homology and promotion of DNA strand exchange. Recruitment of RAD51 promotes sequence-specific synapsis between the targeting DNA and homologous chromosomal sequences, thereby promoting HR. As a proof of concept experiment, we used the hypoxanthine phosphoribosyl transferase (HPRT) locus in mouse ES cells as a targeting site to assess the increase in homologous recombination efficiency. Our results suggest that this technology holds promise in increasing the efficiency of gene targeting.

Poster Board Number: 2228

SCALING-UP THE EXPANSION OF MOUSE EMBRYONIC STEM CELL-DERIVED NEURAL STEM CELLS IN STIRRED BIOREACTORS

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Neural stem (NS) cells are self-renewing multipotent cells present in the developing and adult mammalian CNS. They generate neuronal and glial cells of the developing brain and also account for the limited regenerative potential of the adult brain. Different protocols for NS cell expansion have been developed in both floating and adherent conditions. The propagation of floating cell clusters, called neurospheres, is widely used for NS cell expansion *in vitro*. However, this system shows severe limitations for cell culture like hetrogeneous aggregate composition, diffusion limitations of nutrients, waste accumulation in the cluster centre or preferential astroglial differentiation *in vivo*. The expansion of NS cells on adherent conditions may circumvent most of these limitations. Different sources of NS cells are available, such as the fetal brain, the adult brain or embryonic stem (ES) cells (by *in vitro* differentiation). The propagation of NS cells and their differentiation into mature neuronal phenotypes allows their potential use for treatment of neurodegenerative diseases, neural drug screening and also gene therapy. Although it is anticipated that a large number of cells will be required for those applications, the large-scale expansion and controlled differentiation of ES cell-derived NS (ESNS) cells on bioreactors has not been addressed in detail. This work aims to solve the main biological and technological hurdles that limit this application using as model a cell line of mouse ESNS. mESNS cells are currently cultured under static conditions on tissue culture plastic. However these culture systems are not amenable for large-scale applications since they are limited by a reduced surface area/volume ratio, have a non-homogeneous nature, resulting in concentration gradients (growth factors, metabolites, pH, dissolved O2), and difficult or impossible online monitoring and control. The first step for establishing a mESNS cell spinner-flask culture protocol was a screening of different commercially available microcarriers, to support cell adhesion. To eliminate animal-derived products from the culture, only xeno-free microcarriers were tested, as well as a serum-free culture medium. Selected microcarriers were tested under dynamic conditions, in the spinner-flasks. Superior performance was observed with polystyrene beads coated with a fibronectin fragment. Importantly, high cell viabilities and expression of Nestin, a marker of neural stem/progenitor cells, was retained when cells were cultured for up to 9 days on the microcarriers, in the spinner-flask, confirming that these culture conditions are not detrimental for the cells. The subsequent steps were the optimization of culture parameters. A stirring speed of 60 rpm was found to be optimal and led to a 40-fold increase in cell number after 6 days, with viabilities above 95%. Culture medium feeding is also a critical parameter. The metabolic profile of the culture, in terms of glucose, glutamine and lactate concentrations over time was determined, to avoid nutrient scarcity or metabolite accumulation. The frequency of growth factors supply was also found to be crucial. Finally, microcarrier concentration is also expected to have an important role as it relates with the available surface area. The validation of this bioprocess for mESNS cell expansion will be concluded with the demonstration of the multipotential differentiation of the spinner-flask expanded cells into neuronal and glial cells.

Poster Board Number: 2230

GENERATION OF RAT EMBRYO IN MOUSE BY TETRAPLOID COMPLEMENTATION USING PLURIPOTENT STEM CELLS

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Pluripotent stem cells (PSCs) can expand infinitely while maintaining an undifferentiated state and pluripotency. In rodents, PSCs injected into blastocyst stage embryo can contribute to the embryonic development with chimera formation. Recently, we found that PSCs can contribute to xenogenic embryo development resulting in interspecific chimeras between mouse and rat. These chimeras could survive after birth and the xenogenic PSC-derived cells were present in every tissue we examined. Furthermore, by injecting rat induced PSC (iPSCs) into Pdx1-/- mouse blastocysts, we succeeded in generating rat pancreas in mouse indicating that PSC-derived organ can be generated in xenogenic environment. To extend our findings on interspecific chimeras further, we attempted to generate whole rat embryos in mouse by tetraploid complementation method. Tetraploid embryo produced by electrofusion of 2-cell stage embryo can differentiate into extraembryonic lineages but not into embryo proper. We injected EGFP expressing rat iPSCs into mouse tetraploid embryos and followed up their development in mouse uterus. The results showed that, in gastrulating embryos, epiblast was composed of entirely EGFP-positive riPSC-derived cells and was surrounded by EGFP negative mouse extraembryonic tissues. These embryos could develop until 7 days after embryo transfer (E9.5). They expressed EGFP ubiquitously from head to tail and some embryos reversed and curved ventrally with red blood cells in aortic region. Immunohistochemical and flowcytometric analyses revealed that the embryos were completely derived from injected riPSCs. However, after E11.5, embryonic tissues were degenerated and no embryo survived. These data indicate that riPSC alone can develop in the mouse environment up to E9.5 but not beyond E11.5. The system described here not only provides a powerful experimental tool for understanding of xenogenic barrier in early embryonic development but also an initial step toward the generation of PSC-derived animal in xenogenic environment.

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HETEROGENEITY OF MURINE HEMATOPOIETIC STEM CELL RESPONSES REVEALED BY A MICROFLUIDIC PLATFORM FOR HIGH-THROUGHPUT ANALYSIS OF SINGLE CELLS

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The ability of hematopoietic stem cells (HSCs) to self-renew and differentiate into specialized blood cells is a complex process involving the integration of multiple external cues with the intrinsic molecular state of the cell. Recent *in vivo* analyses of the clonal behavior of cells with functional properties of HSCs (>6 months reconstitution of blood formation) have revealed heterogeneity in their differentiation and self-renewal patterns. To generate matching information and characterize differences in their *in vitro* response to defined stimuli, we monitored HSCs in microfluidic devices containing 1,600 to 6,144 nanovolume culture chambers. These devices were developed to provide automated culture and tracking of thousands of individual non-attached cells over periods of several days. This technology also enables multiple culture conditions to be tested simultaneously, including both constant or temporally varying microenvironments, with an option to perform live cell-surface marker staining on clones without disturbing their spatial position. In this study, we present 4 different applications of this technology in the context of HSC research. First, from growth profiles obtained for hundreds of single NUP98-HOXD13-transduced cells, we demonstrated that only a minority of these cells are highly proliferative and contribute to the overall expansion of bulk cultures. More than 52% of the cells were either destined to die or unable to proliferate. Second, from analyses of the early division kinetics of transduced primary HSCs, we showed that lentiviral vectors carrying a fluorescent reporter gene preferentially transduce and express in those HSCs that begin to divide most rapidly. Third, live immunostaining of the progeny of single HSCs stimulated to divide revealed that continued expression of endothelial protein C receptor (EPCR), a molecule used for murine HSC purification, is associated with delayed entry into cell cycle and longer division kinetics. Finally, we used the unique medium exchange feature of this system to show that the survival and subsequent proliferative activity of very primitive adult hematopoietic cells is independent of Steel factor stimulation during the first 16 h in serum-free culture. Adequate Steel factor stimulation becomes critical for culture times greater than 16 h, as the cells begin to exit their quiescent state. These results illustrate the value of having the combined capabilities of high-throughput live-cell imaging, dynamic control of medium conditions, live immunostaining and selected clone recovery to interrogate otherwise inaccessible mechanisms governing mammalian stem cell growth and fate decisions.

Poster Board Number: 2234

OBSERVATION OF THE MOVING FRONT WHERE MPSCS' STATES SWITCH

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There are a couple of methods to induce differentiation of pluripotent stem cells (PSCs) have been developed so far, such as via generating colonies, forming embryoid bodies, etc. And it has been shown that there were several similarities between PSC's differentiation process and *in vivo* phenomena. Developing the technique to control the key factors of differentiation, such as chemical or protein factors, cell-cell interaction, matrices, physical stresses, etc., would contribute to the reconstruction of *in vivo* situation. We have proposed a microfluidic device for spatially controlled differentiation. The developed device has two compartmentalized channels separated by a polycarbonate porous membrane. Culture media containing differentiation factors are injected into the lower channel where the factors' concentration can be spatially controlled by the laminar flow. Since the factors are transported to the upper channel through the membrane, the cells seeded in the upper channel can be exposed to the differentiation factors with the concentration depending on their positions. We have succeeded in differentiating mouse induced pluripotent stem cells (miPSCs, iPS-MEF-Ng-20D-17 cell line) in a spatially controlled manner in the device by injecting the medium containing retinoic acid (RA) and the one containing leukemia inhibitory factor (LIF). In this study, we further use the device for spatio-temporal control of differentiation factors in microenvironment by changing the exposure time. First we injected two media, one containing RA (10-7 g / mL) and the other containing LIF (1000 Unit), into the device at 1 and 3 µL / min each to induce spatially controlled differentiation. And the cells were exposed to these factors with the concentration controlled by the laminar flow patterns for the durations of 12, 24 and 72 hours. After the exposure, the medium has been changed into LIF containing ones. As a result, when cells were exposed to the differentiation factor for 12 and 24 hours, undifferentiated cells proliferated over the channel. This means that 24 hours exposure was not enough for inducing differentiation. On the other hand, when exposed for 72 hours, differentiation of the cells in the whole area of the channel was induced. This means that 72 hours exposure was enough for inducing differentiation. Moreover we observed the time course of GFP fluorescence of the cells corresponding to the expression level of Nanog. The lateral position in the channel showing the maximum decrease of the fluorescence intensity moved from the RA side to the LIF side. This means that the cells' state rapidly switched at the moving front, as if the differentiation state is propagating from one side to the other along the lateral axis in the channel. This kind of phenomena can only be observed by microfluidic formats, and the technique can be a useful for mimicking *in vivo* situation.

Poster Board Number: 2236

SPECT AND CRYO-IMAGING OF MULTIPOTENT ADULT PROGENITOR CELLS IN A MOUSE MODEL OF GRAFT-VERSUS-HOST DISEASE

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Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) is the only curative therapy for many patients with malignant and hematologic disorders. Graft-versus-host Disease (GVHD) is the most frequent and severe complication of allo-HSCT and limits successful outcomes. Non-hematopoietic stromal stem cells, including multipotent adult progenitor cells (MAPCs) have immuno-regulatory properties, and clinical and pre-clinical data suggest they then can reduce the severity of GVHD. However, the biodistribution of these cells after injection and the immunologic mechanisms behind their protective effects remain to be fully elucidated. Using SPECT and cryo-imaging, we studied the biodistribution of MAPCs in mice. Using a well-described mouse allo-HSCT model, we characterized the biodistribution

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of labeled MAPCs within the first 24 hours of injection. Lethally irradiated, inbred B2D2F1 mice received BM and purified splenic T cells from allogeneic, haplo-identical C57Bl/6 or syngeneic F1 donors. Human MAPCs were either labeled with 99mTc HMPAO (for SPECT imaging) or red quantum dots (Qtracker 625, for cryo-imaging). The MAPCs were then mixed together so that each mouse would receive approximately 0.57 million 99mTc HMPAO-labeled cells and 0.57 million Qtracker-labeled cells. Cells were co-infused via tail vein injection on the day following HSCT. Transplanted mice were subsequently split into 2 groups: those to be sacrificed the same day as the injection (Day 1), and those to be sacrificed the day after the injection (Day 2). Each mouse was subjected to planar scintigraphy and SPECT imaging prior to sacrifice. After the mice were sacrificed, they were frozen and cryo-imaged. Using microscopic resolution, whole mouse cryo-images, we used specialized Bayesian classification software to detect even single stem cells throughout the mouse. Quantitative 3D analysis of SPECT and cryo-images of the Day 1 mice show that the vast majority of injected MAPCs are in the lungs. With cryo-imaging, we detected ≈476,000 of the 570,000 injected fluorescently labeled cells, with 82.6% of detected cells in the lungs. Remaining cells were found in the liver (17.2%) and spleen (0.2%). Signal/cell remained fairly constant. In SPECT images of the same mouse, the majority of the signal from the 99mTcHMPAO-labeled cells was found in the lungs and bladder. Using a calibration from an aliquot of cells, we computed ≈43,000 cells in the lung with SPECT imaging, ≈10% of the number in the same mouse with cryo-imaging. At later time points, we were unable to detect the 99mTcHMPAO-labeled cells in SPECT images. We were, however, able to detect the Qtracker-labeled cells in the cryo images. In Day 2 mice, cryo-imaging detected ≈55,000 cells with an increased percentage in the spleen (83% lungs, 13% liver, and 4% spleen). At early time points, SPECT and cryo-imaging can both track labeled cells in the mouse. Cryo-imaging is better suited for tracking fluorescently labeled stem cells at later time points, where the 99mTc HMPAO label has radioactively decayed and detached from MAPC, as shown by the bladder signal. Because cryo-imaging detects cells, it is independent of a signal/cell calibration as with SPECT.

Poster Board Number: 2238

19F MRI CELLULAR TRACER PRESERVES THE DIFFERENTIATION POTENTIAL OF HEMATOPOIETIC STEM CELLS

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Hematopoietic stem cells (HSC) have numerous applications including immune reconstitution, enzyme replacement, regenerative medicine and immunomodulation. The trafficking and persistence of these cells after administration is a question fundamental to the future of alternative therapeutic applications of HSC. While various contrast agents have been used to track cellular therapeutics, the impact of cellular labels on HSC function has not previously been studied, independently of therapeutic outcome, *in vivo*. Here we describe the labeling of human CD34+ HSC with a novel self-delivering perfluorocarbon (PFC) emulsion. This magnetic resonance imaging (MRI) tracer agent has been found to lack mutagenicity in multiple *in vitro* studies. A comparison of unlabeled and PFC-labeled human HSC in *in vitro* colony forming assays resulted in equal numbers of total colony forming units (CFU), as well as individual CFU types, indicating that labeling did not alter multipotency. In parallel, *in vivo* tests of pluripotency and reconstitution studies in mice with labeled murine BM HSC resulted in equivalent development of CFU-spleen (a measure of HSC progenitor activity) and the reconstitution of both lymphoid and myeloid compartments. The lack of interference in these highly complex biological processes both *in vitro* and *in vivo* following PFC labeling, provides strong evidence that the therapeutic potential of the HSC is likely to be maintained. These data support the safety and utility of using PFC tracers for clinical *in vivo* trafficking of human stem cells.

Poster Board Number: 2240

IN VIVO VISUALIZATION OF CYTOKINESIS IN PLURIPOTENT STEM CELLS AND STEM CELL DERIVED CARDIOMYOCYTES

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The identification and modulation of proliferating cells is critical for stem cell research and regenerative medicine. However, current approaches to quantify cell proliferation are imprecise in particular in tissue types displaying acytokinetic mitosis and endoreduplication such as heart muscle cells. The only definitive proof for proliferative activity followed by cytokinesis in cardiac muscle is the observation of a contractile ring or a midbody prior to abscission. To visualize these cell cycle specific events we developed a new *in vivo* proliferation marker that indicates M-phase in great detail. This was achieved by fusing eGFP to the scaffolding protein Anillin, which is a component of the contractile ring. Anillin accumulates in the nucleus during late G1, S/G2-phase, translocates to the cytoplasm and cell cortex after dissolving of the nucleus, localizes to the contractile ring and the midbody after constriction and is eventually degraded by the proteasome after abscission. Transgenic murine embryonic stem cell (mESC) lines were generated that stably expressed the eGFP-anillin fusion protein under control of the ubiquitous CAG promoter. mESCs of this line displayed a high overlap of eGFP-anillin expression with the mitotic marker Ki-67 in immunofluorescent stainings (96%) and flow cytometric analysis (94-99%), respectively. During differentiation to embryoid bodies the rate of eGFP-anillin positive cells declined with the appearance of postmitotic cells. The fusion protein was detected exclusively in proliferating cells of all three germ layers, thereby demonstrating the high accuracy of this proliferation marker. Transduction with a CAG-eGFP-anillin lentivirus enabled us to compare cell-cycle kinetics of human induced pluripotent stem cells (hiPS) and hESCs via time lapse microscopy and we found significant differences in cell cycle duration. For *in vivo* imaging of proliferation we generated a CAG-eGFP-anillin mouse line which allows tracking of dividing cells during embryonic development and upon cardiac injury at the adult stage. In the embryonic neuroectoderm time lapse microscopy was used to monitor cell-divisions of apical and basal progenitor cells. This new system for visualization of proliferation allows the monitoring and quantitation of cell proliferation *in vitro* and *in vivo* even in tissue types prone to DNA repair, acytokinetic mitosis or endoreduplication. This approach will probably enable us to address open questions concerning the proliferation of heart muscle as well as other tissue types during development and upon injury.

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INTRACELLULAR LABELING OF MULTIPLE CELL TYPES FOR MRI-BASED *IN VIVO* CELL TRACKING

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Cell transplantation is an area of intense investigation especially with respect to stem cell therapy and transplantation studies of embryonic stem cell derived cell-types for treatment of degenerative diseases like Parkinson or heart failure. There is a need to serially image cells after intravenous administration or direct injection into tissue, in order to track migration into the target tissue. Monitoring the location and migration of grafted (stem) cells is essential for understanding their interaction within the host and their therapeutic effects. Therefore, cell tracking is of significant importance for basic research, preclinical evaluation as well as monitoring of early clinical trials applying cell transplantation. Magnetic resonance imaging (MRI) is the most frequently used technique for *in vivo* cell tracking applications due to high resolution of soft tissues, which makes it especially useful for imaging of the brain, muscles or the heart. Detectability of transplanted cells by MRI depends on their contrast characteristics. In order to produce a strong contrast against surrounding tissue, intracellular labeling of cells with iron oxide particles before transplantation has been described. We have established a two component protocol for *in vitro* intracellular labeling of multiple cell types with superparamagnetic iron oxide (SPIO) particles. Contrast particles and loading reagent were optimized for proper *in vitro* labeling of cell lines (NIH-3T3, Jurkat), primary cells (granulocytes, neural progenitors) and stem cells (hematopoietic, mesenchymal and embryonic stem cells) from different species (mouse, rat, human). Highly efficient intracellular labeling of various cell types with contrast particles was proven by prussian blue staining and anti-dextran immunofluorescence analysis. Intracellular labeling neither affected viability nor proliferation. Additionally, similar results with labeled and unlabeled hematopoietic stem cells, mesenchymal stem cells or embryonic stem cells were obtained in CFU assays and upon differentiation into osteoblasts, adipocytes and neurons, indicating biocompatibility of intracellular labeling with contrast particles. In order to evaluate feasibility of the newly developed SPIO particles for magnetic resonance imaging, we analyzed intracellular labeling of neural progenitors after transfection of contrast particles. MRI analyses revealed an *in vitro* detection limit of 250 labeled cells. After transplantation into mouse cortex, intracellular labeled cells were clearly identified with high field MRI scanners. In contrast, extracellular labeling of cells with MicroBead-conjugated antibodies failed to give similar signal intensities. We have developed a new method for *in vitro* intracellular labeling of multiple cell types with SPIO particles without affecting cellular characteristics or fates for high resolution MRI-based cell tracking.

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NEWLY DEVELOPED NANOPARTICLES FOR CELL TRACKING USING MAGNETIC RESONANCE IMAGING

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Purpose: To develop highly magnetic, biocompatible, superparamagnetic iron oxide (SPIO) nanoparticles suitable for *in vivo* tracking of human lymphocytes and CD34+ cells. Introduction: The utilization of magnetic resonance imaging (MRI) to noninvasively monitor cells using SPIO nanoparticles as MRI contrast agents has been one of the major research focuses over the past several years. In particular, the ability to use MRI to image longitudinally offers an invaluable opportunity to track the migration, persistence and distribution of cell-based therapeutics *in vivo* in humans with high resolution on tissue planes. Current approaches for cell labeling have focused on using commercial SPIO nanoparticles with varying success due to differences in size and limited magnetic properties. Therefore, there is a clinical need to develop SPIO nanoparticles that are non-toxic, greater than 50nm in diameter, and produce high MRI contrast. We have utilised SPIO nanoparticles with a silica coating to investigate the labeling capacity and contrast enhancement of blood cells including human lymphocytes and CD34+ expressing cells. Method: Human buffy coats were used to separate and collect lymphocytes and CD34+ cells. A thermal decomposition method was used to synthesise iron oxide silica nanoparticles analysed with transmission electron microscopy (TEM) and x-ray diffraction (XRD). Suspensions of these silica coated SPIO nanoparticles were incubated with isolated peripheral blood mononuclear cells and CD34+ purified cells at 5 µg/ml, 10µg/ml, 50µg/ml and 100µg/ml *in vitro* and then imaged on a clinical MRI 3.0 tesla scanner. T2 relaxation, which is responsible for contrast in T2 weighted MR images, were calculated and iron content performed using atomic absorption spectroscopy (AAS). A 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) based assay was used to measure cell cytotoxicity 24 and 48 hours post-labeling with SPIO silica nanoparticles. Results: Iron oxide nanoparticles coated in silica were made with TEM showing these as monodisperse iron oxide coated in a silica shell with a maximum diameter of 60 ± 7 nm. A high *in vitro* T2 relaxivity of 210.40 mM s was calculated, giving negative MRI signal enhancement of 15%, 45%, 50%, and 75% for concentrations of 5 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml, respectively. The cell cytotoxicity assay showed no toxicity with SPIO silica nanoparticles for concentrations up to 50 µg/ml. Conclusion: This pilot study showed that the newly prepared iron oxide silica nanoparticles can be used for safe and effective cell labeling of human cells. Due to a high T2 relaxivity and possible higher uptake of the nanoparticles, significant contrast enhancement was visualised *in vitro*. The results indicate that smaller number of cells may be needed for MRI detection. Overall, these results provide preliminary evidence that high efficiency biocompatible labels can improve future cell tracking using MRI and SPIO nanoparticles as contrast agents.

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SELF-ASSEMBLING IRON-BASED NANOCOMPLEXES USING THREE FDA-APPROVED DRUGS - FERUMOXYTOL, HEPARIN AND PROTAMINE: IMPLICATIONS FOR STEM CELL LABELING AND TRACKING BY CLINICALLY APPLICABLE MRI

Thu, Mya Mya S.[1], Bryant, Lacy H.[1], Coppola, Tiziana[1], Jordan, Elaine K.[1], Budde, Matthew D.[1], Lewis, Bobbi K.[1], Chaudhry, Aneeka[1], Varma, Nadimpalli Ravi S.[2], Arbab, Ali S.[2], Frank, Joseph A.[1]

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Stem cell-based therapies have become a major focus in regenerative medicine and tumor trials, along with release of cytokines and growth factors to diseased tissues. To further understand the relevant biological and clinical effects of cellular therapies so as to optimize the therapeutic regimens, it is important to develop clinically-applicable imaging approaches that would allow for the monitoring of the temporal spatial migration of stem cells non-invasively. Labeling stem cells with superparamagnetic iron oxide nanoparticles (SPION) allows for monitoring of transplanted or implanted cell behavior by MRI *in vivo*. We previously developed a technique that involves adding FDA-approved dextran coated SPION ferumoxides with protamine sulfate to magnetically label stem cells. However, ferumoxides is no longer manufactured. Currently, Ferumoxytol, a non-dextran-coated USPIO nanoparticle, has been approved for the treatment of iron deficiency anemia in chronic kidney disease. Ferumoxytol alone or in combination with protamine does not label the cells. We developed a novel and straightforward magnetic cell labeling approach that combines three FDA-approved drugs, ferumoxytol (F) (Feraheme®) at (50-100 µg/ml), heparin sulfate (H) at (1-2 IU/ml) and protamine sulfate (P) at (20-60 µg/ml) in serum free media to form self-assembling nanocomplexes that effectively label multiple type of stem cells (Bone marrow stromal cells (BMSC), Neural Stem Cell (NSC) and Hematopoietic Stem Cell (HSC)) and of immune cells (T cells and Monocytes) for cellular MRI. HPF nanocomplexes are incubated in serum free media with cells for 2 hours followed by complete media. Electron Microscopy revealed internalized HPF inside endosomes, confirmed by Prussian blue staining of labeled cells. The average iron content per cell was as follows: BMSC = 2.12+/-0.11 picograms (pg); NSC= 2.8+/-1.19 pg; HSC = 1.33+/-0.01 pg; T-cells= 0.73+/-0.25 pg; and Monocytes= 2.56+/-1.1 pg. Unlabeled cells contained 0.0-0.5 pg/cell and the increase in iron content in the HPF labeled cells was statistically significant ($P<0.05$). There was no effect or toxicity on cellular physiology or function of HPF labeled cells when compared to controls. *In vivo* MRI at 3Tesla detected 1000 HPF-labeled cells implanted in rat brains. HPF nanocomplexes form without chemical modification to the parent drugs and therefore should facilitate the rapid translation of this labeling technique in cell therapy clinical trials.

Poster Board Number: 2248

MAGA AS A NOVEL GENETIC MRI REPORTER FOR MONITORING STEM CELL GRAFTS

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Cell replacement therapy and regenerative medicine hold an enormous potential of curing contemporarily incurable diseases such as diabetes, autoimmune diseases, and neurodegenerative diseases such as Parkinson's, Huntington's, and Alzheimer's. However, one of the obstacles in making clinical translation is the difficulty in long term monitoring of the fate of grafted cells non-invasively *in vivo*. Magnetic resonance imaging (MRI) has unique advantages over the other imaging methods such as positron emission topography (PET) and optical imaging technology in imaging deep tissues with high spatial and temporal resolution. However, implanted cells cannot be monitored with MRI unless a contrast agent, such as cell labeling with magnetic nanoparticles, is used. However, the invasiveness or limited monitoring duration due to the dilution of the contrast agent in the cells has been the problem. The best approach is to develop a transgenic reporter that is capable of generating endogenous contrast effect for MRI. Here, we investigated MagA, a magnetite and magnetosome forming prokaryotic gene, as a novel MRI reporter gene for tracking stem cell grafts *in vivo*. The accumulation of iron as a result of MagA expression may lead to T2 weight MRI contrast as demonstrated previously. MagA gene under a doxcycline promoter was successfully expressed in the mouse embryonic stem cell (mES) line, and characteristics of MagA gene expressed in mES cell have been evaluated through cell grafting, quantitative real-time PCR, and histochemistry. mES cells expressing MagA were successfully grafted in nude mice and formed teratoma at the pace compatible with the AB 2.2 mES cell line. A stable expression of MagA gene was observed in grafted mES cells with significant induction with the doxycycline treatment. Comparing to the wild type, the amount of iron in the teratoma grown from the cells with MagA also increased as the MagA gene was induced with doxycycline by feeding the mouse with doxycycline. These results suggest that MagA can be used as a non-invasive transgenic MRI reporter to track the cell growth and fate after the cell grafting.

Poster Board Number: 2252

TWO CASES OF CONGENITAL CEREBRAL DYSPLASIA TREATED BY AFFINITY UMBILICAL CORD MESENCHYMAL STEM CELLS

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Objective: To explore the clinical efficacy of affinity umbilical cord mesenchymal stem cell (AUC-MSC) in treatment of congenital cerebral dysplasia disease. Methods: Two patients suffering from congenital cerebral dysplasia received AUC-MSC therapy, the stem cells volume is 20ml per patient each time (cell number was 1 × 107), 16ml of which was intravenous, 4ml was given a subarachnoid injection by lumbar puncture way, once per 2 weeks, four-times for a course , each patient was treated totally 8 times for 2 courses. One case of 5-year-old girl was congenital growth retardation

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of motor function, speech impairment, normal muscle tension, no seizures and dance-like movements, brain MRI examination revealed normal cranial structure, mesenchymal stem cells derived from her newborn sister's umbilical cord. One case of 11-year-old boy with congenital growth retardation of motor function, he was born with asphyxia showing spastic disorders especially the lower limbs, difficulty walking, unsteady gait, normal intelligence, high muscle tension; With improved the Ashwoth Standard (MAS), quadriceps was 4 points, gastrocnemius 3; Babinski sign, Oppenheim sign (+), cranial MRI revealed no abnormalities, mesenchymal stem cells derived from his younger brother's umbilical cord. Results: Followed up for 22 months, 5-year-old girl compared with the pre-treatment, got fine activities to improve, reduced the frequency of falling, and got up after falling herself, enhanced immunity, increased physical strength, speech and comprehension had a slight improvement. The 11-year-old boy was followed up for 16 months, his lower limbs muscle tension decreased, walking got well, MAS score: quadriceps was 7 points, gastrocnemius 5. Conclusion: Using affinity UC-MSC treatment of congenital cerebral dysplasia, especially the performance of the motor system in patients with developmental disorders, according the 2 cases of clinical practice, may be one of safe and practical new ways and a good choice.

Poster Board Number: 2254

ADULT STEM CELL APPLICATION IN 21 CASES OF NERVOUS DEGENERTION DISEASE

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Background: Stem cell therapy is very useful in the field of regeneration, there are many reports about traumatic central nerve diseases treated by the therapy, but until now no good way to treat neural degenerative disease. Objective: To study the feasibility of umbilical cord mesenchymal stem cell and autologous purified CD34+ stem cells in neural degenerative disease. Methods: From May 2008 to May 2010, 21 patients with neural degenerative disease were treated with adult stem cells containing autologous purified CD34+ stem cells and umbilical cord mesenchymal stem cells. These patients included 15 cases of motor neuron disease and 6 cases of spinocerebellar ataxia. The 21 patients were divided into two groups with one group including 14 patients received umbilical cord mesenchymal stem cells and the other including 7 patients received autologous stem cells. A volume of 4 ml stem cells (>1.0×107) were administrated into subarachnoid space by lumbar puncture. All of them were treated with the rehabilitation. Results: In 15 patients of motor neuron disease, 8 cases (53%) achieved progress results, 4 cases (27%) obtained markedly effective results, 3 cases (20%) were null and void, and the total effective rate was 80%, before and after treatment the amount of ALS table and the self-assessment questionnaire scores were significantly different (P <0.05); In 6 cases of spinal cerebellar ataxia, 3 cases (50%) achieved the progress results, 1 (17%) case achieved the effect markedly, and 2 cases (33%) were not valid, the total effective rate was 77%. Four of the 21 cases had mild intracranial hypotension headache (after lumbar puncture). Two of the 14 patients with UC-MSC-treated had fever 2h after treatment. The remaining patients had no significant adverse reactions after treatment. Conclusion: Stem cell transplantation may be one of the safe and practical new ways used to treat neural degenerative disease.

Poster Board Number: 2256

AUTOMATED SYSTEM FOR DERIVATION AND EXPANSION OF INDUCED PLURIPOTENT STEM CELLS

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Induced pluripotent stem cells (iPSCs) are adult somatic cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes critical for maintaining the pluripotent properties of embryonic stem cells. Similar to embryonic stem cells, iPSCs can be expanded *in vitro* and differentiated into various clinical functional cell types for research or therapeutic application. During the process of iPSC derivation, potential colonies are picked for expansion and characterization, which are tedious, imprecise and contamination-prone processes. To automate stem cell colony passage and purification, we have developed a bench-top automated system equipped with an identification device, a dissection and transfer apparatus, laminar flow and HEPA filtering system. The system can identify the newly formed colonies from feeder cells and pick up the selected areas for transfer and expansion in a sterile environment. Using this system, some expanded colonies showed pluripotent stem cell characteristics including alkaline phosphatase activity, pluripotency marker expression and pluripotent differentiation ability. In addition to stem cell passage and purification, this system can also be utilized to select differentiated cells derived from iPSC or embryonic stem cells such as neural progenitors. By eliminating tedious manual work, the automated system can improve quantity and quality of iPSCs, providing a more consistent and reliable cells for research or clinical applications.

Poster Board Number: 2258

CLONAL TRACKING OF *MESC* DURING *IN VITRO* DIFFERENTIATION

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Embryonic stem cells are a valuable tool to investigate mammalian development *in vitro*. As a model system they allow to analyse the impact of transgene-overexpression or down-regulation during differentiation. To evaluate whether mESC are composed of a heterogenic population or if every cell gives rise to further developmental tissue we used the unique integration sites of stably integrated lentiviral vectors (LV) as molecular tags for individual mESC to examine the clonal composition during differentiation. Therefore, mESC were clonally marked using an eGFP expressing LV. Transduction of mESC results in 60 - 65.2% eGFP+ cells and eGFP was stably expressed for up to 27 passages. Differentiation of individually tagged mESC was induced by LIF withdrawal. Insertion site analyses by highly sensitive nrLAM (non-restricted-Linear Amplification Mediated)-PCR coupled to next generation sequencing was performed to monitor the clonal composition in ESC, after embryoid body (EB) formation and cardiomyocyte (CM) differentiation. We identified a total of 295 unique integration sites (IS) in the transduced mESC population serving as origin for the generation of EB and CM, 352 IS in EB and 576 IS in differentiated CM. Comparison of IS revealed an overlap of 101 IS in EB and mESC. In addition, 170 IS were found in differentiated CM as well as in mESC or EB. Mapping of IS to the murine genome revealed that in pluripotent mESC IS tagged genes were enriched in functional categories involved in cell movement, embryonic development and cell-to-cell signal-

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ing. In contrast, IS in differentiated EB and CM were preferentially detected in the vicinity of genes contributing to cell morphology, cellular development and cardiovascular disease. These data demonstrate that the clonal contribution of individual mESC clones to differentiation can be monitored *in vitro*. Thorough investigation of clonal dynamics in mESC cultures during differentiation may help to identify subfractions of mESC which are prone to certain differentiation programs.

Poster Board Number: 2260

LABEL FREE MONITORING OF STEM CELL DIFFERENTIATION USING MICROTECHNOLOGY

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The differentiation of embryonic stem (ES) cells into specific progeny is critical for their use as potential therapeutic agents. Monitoring differentiation of living cells would enable the generation of precise cell lineage maps, but current live cell techniques are limited by insufficient markers for all potential ES cell progeny. Our previous results suggest dielectrophoresis (DEP) may provide a quantitative, label-free way to monitor the differentiation of stem cells. In this study, we utilized a microfluidic DEP trapping device to determine the dielectric properties of stem cells at various differentiation stages. We initially clarified the balance of fluidic and DEP forces in our device by measuring the dielectric properties of relatively homogeneous cell lines and found we could derive specific membrane capacitance and crossover frequency values for the cells. We then calculated these properties for stem cells spanning the developmental progression from pluripotent ES cells to terminally differentiated CNS cells. Our results show that specific membrane capacitance and crossover frequency shift as the cells differentiate from one cell type into the next, thus providing a quantitative measure of cell phenotype. This approach can be applied to any stem cell population to enable live cell detection of lineage-specific differentiation.

Poster Board Number: 2262

SEMANTIC WEB MODELING FOR INNOVATION IN TRANSLATING STEM CELL SCIENCE INTO REGENERATIVE MEDICINE - HARNESSING DATA FLOWS IN THE ICASM (INTERNET CLOUD, APPS, AND SOCIAL MEDIA)

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The innovative power of data flows through the Internet cloud, mobile apps, and social media (the ICASM) extends to translating stem cell science into regenerative medicine (SCS-to-RM). In this respect, tens of thousands of web, blog and microblog posts about stem cells appear daily with the potential for some portion of this Amazonian flow to accelerate and advance the SCS-to-RM process. The great preponderance of these posts, however, comprises background noise. This presents two challenges to the SCS-to-RM community: 1) developing a means by which to filter from the noise the few signals with innovative power; and 2) applying this power to building and continually improving models of the SCS-to-RM process for purposes of spurring the scope, speed and safety of progress. We hypothesize that semantic web technology can be employed to address these two challenges. By semantic web technology we mean the tools - sometimes called Web 3.0 - under development over the last two decades under the auspices of organizations such as the W3C Consortium and the IEEE. We test the SCS-to-RM Semantic Web Modeling Hypothesis with an experiment consisting of the following four iterative steps: 1) designing a semantic web ontology for building and maintaining SCS-to-RM models (the SCS-to-RM Ontology); 2) building an SCS-to-RM model from the SCS-to-RM Ontology based on the plethora of sources in the public domain; 3) developing and applying a series of algorithms for signaling the potential need to alter the SCS-to-RM model based on input from a subsequent set of blogs, microblogs and Internet alerts; and 4) assessing the potential of these changes in the model for spurring innovation. To pursue Step 1 we have further developed the ontology proposed for the Stem Cell Information Technology Accelerator Platform (SCITAP) presented in a poster at the ISSCR 2009 Annual meeting. To pursue Step 3 we have further developed the text analysis algorithms presented in a poster at the ISSCR 2010 Annual Meeting. Preliminary results suggest that semantic web tools can be fruitfully applied to ICASM data flows in the pursuit of model building for improving the process of translating stem cell science into regenerative medicine. As in other fields, these results raise the intriguing question as to the impact of semantic web modeling on the evolution of the nomenclature and syntax used to describe the SCS-to-RM process and on further development of web-based tools such as clinicaltrials.gov. These results also suggest the value of forming a working group under the auspices of the ISSCR, the W3C Consortium, and/or the IEEE to pursue a community-wide initiative to develop an SCS-to-RM Semantic Web platform.

Poster Board Number: 2264

SAFETY DATA EXCHANGE IN COLLABORATIVE ARRANGEMENTS FOR TRANSLATION, DEVELOPMENT AND IMPLEMENTATION OF STEM-CELL BASED REGENERATIVE MEDICINE

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Under the watchful eye of government regulators, developers and providers of regulated medical products, such as drugs, biologics and medical devices, engage in unceasing effort to monitor and maintain the safety of these products. Contributing to this need for incessant attention to safety, each new generation of medical technology creates new demands on this effort. The translation of stem cell science into regenerative medicine and the development and implementation of regenerative medicines and therapies based on stem cell science and technology offer no exception to this rule. And with the clinical studies of stem cell-based therapies recently allowed by the U.S. FDA to proceed, the topic of safety in clinical trials and eventual worldwide implementation of regenerative medicine based on stem cell technology takes on added importance. As is true for regulated medical products in general, the safety profile of any proposed regenerative medicine gathers mass as it traverses the pathway from its conception and translation to its wide, society-wide adoption and implementation. At each waypoint along this traverse, various aspects of product and therapeutic safety must satisfy a set of increasingly complex biological, medical, cultural, ethical, economic, and technology and business development factors. The study reported here begins with the axiom that the regenerative medicine industry must efficiently and effectively set forth and follow a clear, well-defined set of specifications for each of these factors against the backdrop of two dimensions: the safety aspects of regenerative medical products in general; and the safety aspects of any specific regenerative medical product under translation and development. In addition, the study reported here also sets forth as an axiom the fact that most regulated medical products traverse the conception-to-translation-adoption pathway as a result of the collaborative efforts of several entities. These two axioms thus lead to the need for the regenerative medicine industry to begin to define the contours of safety data exchange in the context of collaborative translation, development and implementation of regenerative medicines. The study reported here focuses on defining these contours. In particular, the study explores whether general rules regarding safety data exchange in the context of collaborative development and implementation of regulated medical products need to

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be customized for regenerative medicine. By exploring this topic this paper seeks to propose a library of contractual provisions that the regenerative medicine community can use in collaborative arrangements focused on the translation, development and implementation of stem cell-based regenerative medicine. Among the potential benefits of such a library would be its contribution to the fostering of an industry-wide safety data infrastructure, the provision of one or more tools to support safety training, and lower transaction costs.

Poster Board Number: 2266

SYSTEMATIC IDENTIFICATION OF TRANSCRIPTION FACTORS CAPABLE OF INDUCING CELLS OF INTEREST

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A mammalian body consists of two hundred types of cells. The molecular mechanism making these differences is regulated by transcriptional network, which is organized by interactions between transcription factors and epigenetic factors. Recent reports have shown that forced expression of a set of transcription factors can induce an intended type of cells by transdifferentiation (e.g. iN and iCM cells), suggesting that some of transcription factors may be able to recruit epigenetic factors on a large number of gene to eventually organize transcriptional network. It is hoped that further identification of such transcription factors in various types of cells will promote the understanding of transcriptional networks and cellular differentiation. Here we show the novel screening system to identify transcription factors with the cellular induction activity. By using this system in an example type of cells, we identified several transcription factors capable of inducing the cells with similar characteristics to that used in the screening. We hope this system to be useful to study how transcriptional network is organized in a cell.

Poster Board Number: 2268

EFFICIENT HOMOLOGOUS RECOMBINATION IN ZSCAN4C EXPRESSING CELLS

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One of the unique features of mouse ES cells is the high efficiency of homologous recombination (HR), which has been widely used to carry out gene targeting in mouse ES cells. In higher eukaryotes, except for a chicken DT40 cell line derived from B lymphocytes, the HR occurs relatively infrequently. The mechanism by which the HR is facilitated in mouse ES cells is not well understood. Recently, we have reported that Zscan4 (zinc finger and SCAN domain containing 4) shows unique expression pattern and function in ES cells. Zscan4 was originally identified as late 2-cell stage specific gene, and also detected in undifferentiated ES cells for their spotty expression pattern (~5% positive cells in ES cell cultures). Zscan4-positive population in ES cells shows the elongation of telomeres and the suppression of genomic sister chromatid exchange activity, suggesting possible involvement of Zscan4 in DNA repair and recombination systems. These observations prompted us to investigate the HR events in Zscan4-positive cells. Here we show that the HR occurs more frequently in Zscan4+ ES cells than in Zscan4- ES cells. For example, using a Tamoxifen-inducible CRE-ERT2 system in ES cells carrying a floxed LacZ, we found 5-times enrichment of LacZ+ cells after transfection of promoter-less gene targeting vector and subsequent drug selection compared to cells after transfection of a random integrating vector and drug selection. We also found more frequent HR events in the ES cells carrying a Zscan4-expressing transgene. These data suggest a possibility of enhancing HR frequency by manipulating Zscan4 expression. This research was supported entirely by the Intramural Research Program of the NIH, National Institute on Aging.

Poster Board Number: 2270

DEVELOPMENT AND OPTIMIZATION OF THE ON CHIP MICROCHAMBER ARRAY PCR AND RT-PCR FOR STEM CELL ANALYSIS

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The miniaturization of analytical devices by semiconductor microfabrication technique is attracting many scientists for the application to the medical and bioanalytical fields, such as genetic analysis, clinical diagnostics, drug screening, and environmental monitoring. Especially, the detection sensitivity of amplified DNA at a trace volume polymerase chain reaction (PCR), when compared with the normal scale, was expected to become easy. If the same number of DNA was amplified from the same copy template, DNA concentration per unit volume would be higher at the trace volume. These merits will be important to analyze between single cell characteristics and gene expression. However, miniaturized on-chip DNA amplification devices have serious problems, which are cross-talk contamination between chambers and rapid drying of the trace volume PCR solution at nano scale volume. Then we have developed a new method employing an oil layer on the microchamber instead of glass or plastic film covers. The sample DNA mixture to be amplified was introduced into each of mirochambers of the array by using nano-liter dispensing system through an oil layer. Thus, the feasibility of our microchamber array was further improved by using TaqMan fluorescence PCR. The quantification of initial DNA concentration present in a microchamber was achieved from 0 to 12 copies per chamber, not only by monitoring the real-time fluorescence intensity but also by observing the end point fluorescence signal. Additionally, the target specific DNA amplification from a single chromosome was also succeeded using our microchamber. In this report, we will introduce a microchamber array chip for DNA amplification from a single cell and showed our results of the trace volume PCR amplification of a target DNA in the microchamber array chip. Then we also applied to on-chip RNA detection by through the DNA amplification from mRNA (RT-PCR). We fabricated the silicon microchamber array chip, which had 50 nL volume of each chamber. The some kind of condition of PCR solutions, which contained genomic DNA as a template, specific primers and fluorescence probe, were distributed into the microchambers using nano-liter dispenser. After on chip PCR amplification, we found that the polymerase concentration was needed 10 times comparing with the normal condition. Other hands, we developed the novel tweezer-type probe devices for the manipulation of the single cell. Then, the single cell (fetal blood cell), of which was fixed on the slide glass, was collected using tweezer probe. The collected single cell was released into the microchamber. After PCR amplification, positive signals could be obtained from 13 microchambers (46.4 %). The some kind of condition of RT-PCR solutions, which contained total RNA as a template, specific primers and fluorescence probe, were distributed into the microchambers using nano-liter dispenser. After on chip RT-PCR amplification, we found that the reverse transcriptase was needed 1 times normal condition, however, the polymerase concentration was needed 10 times comparing with the normal condition to detect the difference of fluorescence. In the future, it is expected that the analysis of relation between single cell and gene expression could become possible by the established method.

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TRANSGENIC EXPRESSIONS OF PLURIPOTENCY-RELATED TRANSCRIPTION FACTORS IN COMMON MARMOSET ES CELLS BY SITE-SPECIFIC TRANSGENESIS

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Non-human primate embryonic stem (ES) cells have vast promise for preclinical studies. Genetic modification in non-human primate ES cells is an essential technique for maximizing the potential of these cells. Common marmoset, one of the New World monkeys, has several advantages as an experimental primate. These include their small size (250 to 400 g), ease of breeding, and high reproductive efficiency (average pups/year from a pair, 4). These characteristics are favorable for preclinical studies. However, transgenesis and gene targeting in common marmoset ES (cmES) cells remain difficult mainly because of the obscurity of cell cultures and the low transfection efficiency. Also, the fact that transgenes are often silenced in primate ES cells is another major problem. Recombinase-mediated cassette exchange (RMCE), a transgenic strategy using recombinase activity, enables efficient site-specific integration of a transgene efficiently by inserting a cassette flanked by a pair of heterospecific lox sites into a genomic locus in advance. This method is highly advantageous relative to conventional transgenesis using non-homologous end joining because of its predictable, reproducible and stable expression from a single copy transgene. Therefore, this transgenic strategy is feasible for research using primate ES cells. To establish the site-specific transgenic system using RMCE in primate ES cells, we inserted the enhanced green fluorescent protein (EGFP) gene with heterotypic lox sites into the β-actin (ACTB) locus of the cmES cells using gene targeting. The resulting knock-in ES cell line, BR29, expressed EGFP stably under the control of the endogenous ACTB promoter. Using inserted heterotypic lox sites, we demonstrated Cre RMCE and successfully established a monomeric red fluorescent protein (mRFP) knock-in cmES cell line. Next, to establish various transgenic cmES cell lines by RMCE, Cre expression plasmid and donor plasmids that express pluripotency-related transcription factors NANOG, KLF2, KLF4 or KLF5 were cotransfected to BR29 by lipofection. After drug selection, the resistant colonies were obtained and expanded. These transgenic cmES cell lines showed stable expression of each transgenes over several passages. Therefore, this transgenic strategy is expected to be useful for preclinical studies and stem cell biology using transgenes in the common marmoset.

Poster Board Number: 2274

PREDICTIVE TOXICOLOGY APPLICATIONS OF HESC-DERIVED CELLS

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Predictive toxicology is a critical step in drug development and the evaluation of drug safety. Most currently available models of predictive toxicology are insufficient in identifying toxic compounds. The use of human cells in predictive toxicology screening allows for better correlation of human responses to drug products than traditional animal models, which are limited due to species differences. hESCs represent a reliable and scalable source of differentiated human cell types, that are otherwise difficult to obtain, for cell based assays. California Stem Cell manufactures three high purity hESC-derived cell products in 96-well and 384-well screening plates for use in predictive toxicology. NeuroPlate™ contains functional live neuronal progenitors (NPs), MotorPlate™ contains functional live motor neuron progenitors (MNPs) and CardioPlate™ contains live cardiomyocyte progenitors (CPs). Here we demonstrate the use of these plates in predicting toxicology from lead compounds in development. The use of our high throughput cellular products during the early phases of drug development could lead to safer pharmaceuticals and reduced attrition, potentially saving millions in overall development costs.

TISSUE ENGINEERING

Poster Board Number: 2276

ISOLATION, CLONAL EXPANSION AND GENETIC PROFILE OF HUMAN ADULT EPITHELIAL STEM CELLS FROM THE CELL RESTS OF MALLASSEZ

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Precious little success has been made in the area of adult, genetically unmodified, non-cancer-derived Human Adult Epithelial Stem Cells (hEpiSC) or Progenitor cells despite progress with the human mesenchyme and hematopoietic component or with fetal murine epithelial stem cells from various organs. Isolating human adult hEpiSCs and testing their pluripotency and progenitor capabilities is complicated by the origin of relevant tissues in internal organs. Tissue culture of viable, human epithelial stem cells from more accessible sources such as the epidermis has been hindered by anoikis, an apoptotic cell-death triggered by the loss of cell attachment. Consequently, most tissue-specific hEpiSCs have been identified in tumors, at a point in which they cause cancer and are of no use to the desired end-point of stem cell research: therapeutic application. We report a major advance, as we have been able to clonally isolate, characterize and maintain in a xeno-free, feeder-layer free tissue culture system, epithelial cells with stem-like pluripotency. Our cells originate in the oral cavity as a by-product of orthodontic removal of wisdom teeth, a procedure applied to 99% of dental patients. Our hEpiSC's come from the Human Epithelial Cell Rests of Mallassez (hERM) cells, found around teeth throughout adult life and represent a potential accessible homologous donor stem cell source. Our method can find general application in the isolation of unmodified adult epithelial stem cells. Human integrin-a6+ve cells as well as an Lgr5+ve subpopulation are derived by fluorimetric analysis (FACS). Our sorted populations can grow organoids and express the markers of pluripotency, oct-4, nanog and Sox2. They maintain the hERM growth profile *in vitro*. The genetic profile of Lgr5+ve cells shows increased expression of genes associated with an undifferentiated stem cell phenotype. hEpiSC can generate mineralized tissue *in vivo* when co-seeded on scaffolds with Human Dental Pulp Stem Cells (hDPSC) and grafted subcutaneously in the nude mouse. In grafts with co-seeded hDPSC and hEpisC, epithelioid organ structures appear (6-weeks post-implantation) followed by mineralization (ten weeks post implantation). TEM shows crystal mineral depositions characteristic of both cementum and enamel as well as profiles of cementoblast and ameloblast-like cells. hEpiSC cells alone can give rise to contained acinar, secretory epithelial-like structures but no mineralized tissue. No differentiation into columnar epithelial cells, cementoblast -like cells and formation of mineralized tissue is seen in hERM or hDPSC alone controls supporting the view that mesenchymal-epithelial signaling is required for mineralized dental tissue differentiation. Human origin of cells in grafts is verified by HuNu antibody immunofluorescence. Tooth organ regeneration is envisioned by direct transplantation of stem cells or progenitors in the jaw *in vivo* or by implantation of the *in vitro* engineered tooth. More than cosmetic application tooth organ regeneration is needed for patients

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of trauma and severe tooth abnormalities such as Amelogenesis Imperfecta. This is the first report showing regeneration of human enamel and human cementum *in vivo* and. Differentiation protocols to address the question of hERM in broad application as hEpiSC are being evaluated. It is conceivable that the same pluripotent cell used for tooth regeneration might be used for skin, or kidney or liver regeneration.

Poster Board Number: 2278

COMPRESSION-INDUCED ALIGNMENT OF HUMAN MESENCHYMAL STEM CELLS IN COLLAGEN CONSTRUCT

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Cell alignment is an important design parameter in tissue engineering. Using a micromanipulator-based loading device, we delivered compression loading to human bone marrow derived mesenchymal stem cells (MSCs) in collagen constructs and investigated the alignment response of these cells upon loading. We demonstrated that hMSCs preferably aligned along the compression loading axis. Moreover, the matrix factor, particularly the collagen concentration, interferes with the mechanoresponse of hMSCs. Specifically, the compression-induced hMSCs alignment and elongation is collagen concentration-dependent, with moderate collagen concentrations giving the optimal responses. Our results also suggest the importance of integrin beta 1 in mediating the mechanoresponses. Moreover, hMSCs respond to compression by exhibiting morphological change in actin organization. Furthermore, compression may induce the expression of matrix adhesions other than focal adhesion and may stimulate the maturation of matrix adhesions.

Poster Board Number: 2280

HUMAN FALLOPIAN TUBE MESENCHYMAL STEM CELLS ENHANCE BONE REGENERATION IN A XENOTRANSPLANTED MODEL

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The treatment of osteoporosis, which affects about 70% of women over 50 years of age, has been the focus of many researches. We have recently reported that human fallopian tubes, which are discarded during surgical procedures of women submitted to sterilization or hysterectomies, are a rich source of mesenchymal stem cells (htMSCs). It has been previously shown that stem cells may be useful in enhancing the speed of bone regeneration. This prompted us to investigate if htMSCs may be useful in the future treatment of osteoporosis since they present a pronounced capacity for osteogenic differentiation *in vitro*. Based on this prior knowledge, our aim was to evaluate, *in vivo*, the osteogenic capacity of htMSCs to regenerate bone through an already described xenotransplantation model: nonimmunosuppressed (NIS) rats with cranial defects. htMSCs from five 30-50 years old and fertile heathy women were used in this experiment. They were characterized by flow cytometry and for their multipotenciality *in vitro* (osteogenic, chondrogenic and adipogenic differentiations) prior to the transplantations. Two symmetric full-thickness cranial defects (5 x 8 mm) on each parietal region of seven NIS rats were performed. The left side (LS) of six animals was covered with CellCeram (Scaffdex) only, and the right side (RS) with the CellCeram and htMSCs(106 cells/scaffold). In one control animal, the RS had CellCeram only and nothing was added at the left side (controls) to ensure, that the provoked cranial defect does not regenerate spontaneously. Animals were euthanized at 30, 60 and 90 days postoperatively and cranial tissue samples were taken for histological analysis. After 90 days we observed neo-bone formation in both sides. However, in animals euthanized 30 and 60 days after the procedure a neo-bone formation with a mature bone was observed only in the right side. PCR analysis confirmed the presence of human DNA only at the right side of the skull. This result was confirmed by immunofluorescence specific for human nuclei. Our results show, for the first time, that htMSCs can be used successfully to enhance bone regeneration *in vivo*, opening a new field of study for future treatments of osteoporosis with women autologous stem cells.

Poster Board Number: 2282

IDENTIFICATION OF SPECIFIC POPULATION OF HUMAN SALIVARY ACINI AND THEIR PROGENITORS USING MESENCHYMAL STEM CELL MARKERS

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Xerostomia results from irreversible destruction of salivary parenchyma in patients afflicted with Sjogren's syndrome or who received radiotherapy for head and neck cancer. The development of an artificial salivary gland device or stem cell-based therapy would benefit these patients. Accordingly, we need to expand acinar cells and/or their progenitors. We aim to identify and characterize human salivary gland (huSG) stem/progenitor cells, and to track them in a duct-ligated parotid gland in a rabbit model. Human salivary tissue (n=12) was tested for the expression of mesenchymal stem cell markers: Stro-1, CD146, CD106, CD44, CD166, CD90, CD105 using immunofluorescence technique. Moreover, CD44 expression was tracked in rabbit parotids after duct-ligation (n=30). In huSG tissue, 100% of serous acini expressed the mesenchymal adhesion-related stem/progenitor cell marker CD44, while 100% of mucous acini expressed CD166. In rabbit controls, all serous acini also expressed CD44. However upon ligation, CD44 expression increased in both acini and ducts at day 1, 7, 14 and only in duct-like structures at day 30 and 60 post-ligation. These CD44 stem/progenitor cells are more resistant to atrophy caused by duct-ligation than the differentiated cells. This duct-ligation model might be used to isolate a greater number of stem/progenitor cells while CD44/CD166 could be used either to isolate stem cells or differentiated serous/mucous acini from human salivary glands. The ability to identify and isolate huSG stem/progenitor cells would provide a cell-based therapy for the treatment of xerostomia.

Poster Board Number: 2284

DYNAMICALLY PRIMING HUMAN FETAL MESENCHYMAL STEM CELLS FOR BONE TISSUE ENGINEERING APPLICATION—A COMPARATIVE STUDY OF DIFFERENT BIOREACTOR SYSTEMS

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Bioreactors provide dynamic culture conditions for efficient exchange of nutrients and the mechanical stimulus necessary for generation of effective tissue engineered bone grafts (TEBG). We have shown that biaxial rotating (BXR) bioreactor-matured human fetal mesenchymal stem cell (hfMSC) mediated-TEBG can heal rat critical sized femoral defects. However, it is not known whether optimal bioreactors exist for bone TE (BTE) applica-

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tions. We systematically compared this BXR bioreactor with three most commonly used systems: Spinner Flask (SF), Perfusion and Rotating Wall Vessel (RWV) bioreactors, for their application in BTE applications. The BXR bioreactor achieved higher levels of cellularity and confluence (1.4-2.5x, $p<0.05$) in large 785mm3 macroporous scaffolds not achieved in the other bioreactors operating in optimal settings. BXR bioreactor treated-scaffolds experienced earlier and more robust osteogenic differentiation on von Kossa staining, ALP induction (1.2-1.6x, $p<0.01$) and calcium deposition (1.3-2.3x, $p<0.01$). We developed a MicroCT quantification method which demonstrated homogenous growth of hfMSC in BXR bioreactor-treated grafts, but not with the other three. BXR bioreactor enabled superior cellular proliferation, spatial-distribution and osteogenic induction of hfMSC over other commonly used bioreactors. In addition, we developed and validated a non-invasive quantitative microCT-based technique for analyzing neo-tissue formation and its spatial distribution within scaffolds.

Poster Withdrawn

Poster Board Number: 2288

SCANNING OF BIOMATERIAL COMPOSITION AND MICROSTRUCTURE FOR HUMAN ADULT STEM CELLS ASSOCIATION AND OPTIMIZATION OF LARGE BONE DEFECTS REGENERATION

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Although smart biomaterials have great potential to be used in bone reconstruction surgeries, transplantation of autologous bone is still the golden standard in bone regeneration procedures. An ideal scaffold to support bone formation must have osteoconductive and osteoinductive properties which may be enhanced through the association between the biomaterial and stem cells harboring osteogenic potential. Furthermore, there are a number of recent reports showing that cellular behavior can be orchestrated by biomaterial properties such as surface topography, macro and microstructure, composition and elasticity. In this study we screened seven biomaterials with different properties to evaluate its effectiveness to heal rat calvarial critical size defects when associated with human dental pulp adult stem cells (hDPSCs). The immunophenotype characterization of these cell populations showed that they stained positively for mesenchymal cell markers (anti-CDs 29,73, 90 and 105) but were negative for the hematopoietic (anti-CDs 34 and 45) and endothelial (anti-CD 31) ones. We attested and quantified the *in vitro* osteogenic potential of these populations through cell culture treatment with osteogenic medium and by alkaline phosphatase and alyzarin red staining. Cells (10e6) were associated to 4.5 mm discs of five commercially available and two in-house developed biomaterials: Geistlich Bio-Oss Collagen (Geistlich Biomaterials), BioGen, BioGen Mix Gel, Biocollagen Gel (Bioteck s.l.r.), CellCeram (Scaffdex Oy), 50% hydroxyapatite (H.A.) / 50% βTricalcium-phosphate (β-TCP) and 100% β-TCP (developed in-house). *In vivo* ossification was accessed using a rat (Wistar) model of paired critical size defects. Control groups were performed transplanting the biomaterials free of cells. We compared experimental and control groups healing 45 days after surgery through histological hematoxylin / eosin staining. We observed a significantly enhanced osteogenesis in experimental groups belonging to three materials: 100% β-TCP, Biocollagen Gel and CellCeram. We detected differences in Osteoblastic differentiation process (first phase of the osteogenic differentiation) in the 100% β-TCP (300 um average pore diameter, 70% porosity) experimental groups with formation of bone islands primordia, single mature bone island formation in the Biocollagen Gel (equine collagen material) experimental groups and generalized mature bone islands formation with broad osteocyte distribution in the CellCeram (60% H.A./40% β-TCP, with 200-400 um of pore diameter and 80% porosity) experimental groups. The best regeneration in this large bone defect model was achieved by association of hDPSCs to CellCeram discs and quantification of total bone island area showed that bone formation was enhanced 4 to 7 times in experimental groups when compared to control groups. In this work we present data supporting successful association of human adult stem cells to ceramic and collagen materials with specific structure and composition to heal large calvarial bone defects. We also suggest that synthetic biomaterials associated with stem cells are a bona fide alternative to autograft therapy to heal critical-size bone defects.

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Poster Board Number: 2292

SELF-ASSEMBLING HYDROGELS FOR 2D AND 3D HUMAN STEM CELL CULTURE

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Introduction: Stem cells isolated from fat tissue are increasingly used in regenerative medicine. Their easy accessibility, differentiation potential and immunomodulatory properties makes them ideal cells for the restoration of damaged or lost tissue. Hydrogels can function as multifunctional scaffolds in various approaches of tissue repair (TR), either as a filler, a slow-release depot for growth factors or a delivery vehicle for (stem) cells. Hydrogels can be prepared from natural ECM proteins or synthetic materials. The advantage of using synthetic materials is that there are no batch-to-batch differences, they have a defined composition and are in general easy to manipulate. Our synthetic self-assembling gels display a nanofibrous structure resembling the *in vivo* ECM structure of collagen fibers. In addition, they allow for easy functionalisation with ECM peptides and cell encapsulation. Therefore, we aim at developing novel (stem) cell-supporting coatings and hydrogels for 2D and 3D cell culture *in vitro* and for (stem) cell-delivery *in vivo*. Materials & Methods: Coatings and hydrogels were prepared from different types of gelator molecules. All gelators share a common core structure consisting of a 1,3,5-cis cyclohexane tricarboxylic acid core. The rigid-core predisposes the arms towards self-assembly resulting in fiber formation and gelation. Gelators with different cell-adhesive properties were obtained by conjugating different peptidic arms to the three arms of the core structure. The self-assembling character of the gelators allows for easy encapsulation of structures like micelles or cells. Characterization of the cells on the coatings and in the gels is done by assessing 1) the morphology of the cells by light microscopy and staining 2) the viability of the cells by staining and biochemical assays and 3) gene expression by PCR. Results & Discussion: The gelators were either applied as 2D-coatings or as 3D-hydrogel matrices in well-plates. The growth of human adipose-derived stem cells (ADSCs) on and in scaffolds of these gelators was studied. Cellular function could be maintained on the coatings and in the hydrogels; the ADSCs survived and proliferated on the coatings and in the hydrogels. In addition, our coatings also allowed for differentiation of ADSCs along multiple lineages, e.g. adipogenic, osteogenic, chondrogenic and neuronal differentiation as shown by staining for cell specific markers and gene expression. These studies showed that our gelators are suitable materials for 2D as well as 3D cell-culturing of ADSCs. Conclusion: Stem cell-culturing on 2D-coatings or in 3D-hydrogels showed that our materials are suitable scaffolds for the growth and differentiation of ADSCs. Preliminary results showed that also cardiac and neuronal cells derived from human ESCs were growing well on our coatings and in our gels. Therefore, our materials offer unique possibilities for the development of stem cell-seeded scaffolds for the regeneration of damaged or lost tissue.

Poster Board Number: 2294

HUMAN STEM CELL-BASED ENGINEERING OF ADIPOSE TISSUE: IMPACT OF ADIPOCYTES ON ENDOTHELIAL CELL CAPILLARY FORMATION

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Various adult stem cell populations are available for tissue engineering applications. Adipose-derived stem/stromal cells (ASCs) are emerging as important multipotent cells for the reconstruction of diversified tissues. We have used human ASCs as building blocks for the production of tissue-engineered connective and adipose tissue substitutes. Considering the importance to achieve rapid revascularization after implantation of thick tissue grafts, we engineered tissues comprising an *in vitro* network of capillaries. We thus report the production of human "endothelialized" reconstructed tissues devoid of exogenous matrix components. The self-assembly approach of tissue engineering was used in combination with ASCs to produce connective and adipose tissue substitutes. This method stimulates cells to organize their own extracellular matrix environment resulting in manipulatable connective sheets. By including an adipogenic differentiation step, adipose sheets can be produced. Thicker tissues are obtained by layering multiple cell sheets. Addition of human microvascular endothelial cells (HMVECs) prior to sheet superposition resulted in the formation of capillary-like structures within the connective and adipose constructs. Immunolabelings and confocal analyses revealed a network of PECAM-expressing capillaries only within tissues enriched with HMVECs. In comparison to connective tissues, adipocytes impacted on the percentage of PECAM+ structures featuring a well-defined lumen on tissue sections (2-fold increase) and a tendency towards 20% greater lumen diameter. This higher number of capillary-like structures was correlated with an increased secretion of many pro-angiogenic molecules from adipose constructs, namely a 15-fold increase in leptin level, 3.3 fold for angiopoietin-1, as well as 2.0 fold for both HGF and VEGF as assessed by ELISA assays. Finally, a short-term study indicated that endothelialized adipose constructs implanted onto nude mice may promote revascularization within the first 7 days after grafting. In conclusion, the use of autologous adult stem cells combined with endogenous human matrix elements make these tissue-engineered adipose tissues unique and natural substitutes for future clinical applications.

Poster Board Number: 2296

INVESTIGATING THE ROLE OF VARYING MOLECULAR WEIGHT OF HYALURONIC ACID IN THE PROLIFERATION AND DIFFERENTIATION OF MOUSE ES CELLS, EB3 *IN VITRO*

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Recently there has been much focus on not just regenerative biomaterials alone, but also exploring their potential as components of a 'niche' in the area of stem cell research. One such biomaterial that holds promise to serve as a 'niche' material is Hyaluronic Acid (HA), which can support controlled self-renewal and differentiation of human ES cells. However the properties of HA and its effects on cellular environments can vary depending upon its molecular weight (4000 Da -3 million Da) as shown by us. Low MW HA is more angiogenic versus high MW which is non-thrombogenic. Such differences in effects due to varying MW of HA are generally mediated via HA receptors such as CD44 and RHAMM. We attempted to investigate the effects of varying MW of HA on mouse ES (EB3) cell proliferation and differentiation *in vitro*. HA of high MW (3 million Da) and of low MW (6.4 KDa) was UV-crosslinked using the photocrosslinker 4-Azidoaniline and patterned onto cell culture cover slips (0.2% w/v). EB3 were seeded (10,000 cells) onto these micropatterned HA surfaces and control gelatin coated dishes and cultured upto 5 days. Immunohistochemistry was used to detect the presence of Oct3/4, CD44 receptor and HA uptake into EB3. ALP staining was used to assess the levels of differentiation, and WST-1 was used to measure cell proliferation. RT-PCR was employed to detect mRNA levels of GATA-4 and Oct3/4 levels within samples. WB was used to assess the levels of phospho- and total STAT-3 proteins. To explore the role of CD44 in uptake and metabolism of HA by EB3, we cultured cells by adding CD44 antibodies and explored the uptake of HA, and resulting effects on cell proliferation and differentiation when cultured on HA. Cells adhered to all wells receiv-

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ing HA, but formed flat spread out colonies only on gelatin, immobilized LMW HA and to some extent in wells that received soluble LMW HA. All colonies appeared undifferentiated as detected by ALP-1 staining. Oct3/4 was expressed by cells grown on all substrates except immobilized HMW HA. WST-1 assay showed that cells proliferated to a maximum extent and equally on gelatin, immobilized LMW HA and in soluble LMW HA compared to others. Reduced GATA-4 mRNA expression in cells cultured on LMW HA (both immobilized and soluble), in soluble HMW HA and on gelatin controls confirmed the undifferentiated state of EB3 cells on these substrates. Increased protein expression of phospho-STAT3, which induces the expression of Oct-3/4 and maintains the undifferentiated state of EB3 was detected in cells cultured on all substrates except on immobilized HA. For the very first time, we demonstrated the presence of CD44 receptors on EB3, which when blocked by adding CD44 antibodies caused reduction in HMW HA, but not LMW HA uptake. This also led to decrease in cell proliferation, and increasing levels of differentiation in EB3 cultured on or with HMW HA. However for cells cultured with LMW HA in the presence of CD44 antibodies, these trends were not affected. Suspecting the involvement of p42/44 MAPK in this HA-CD44 interaction, we blocked the CD44 receptors on EB3. In cells cultured on or with HMW HA, blocking CD44 lead to reduction in the levels of phospho-p42/44 MAPK. This was however not the case for EB3 cultured with CD44 antibodies on LMW HA, wherein the levels of phospho-p42/44 MAPK remained unaffected. Probably there is another pathway, other than via CD44 (maybe RHAMM) which is responsible for LMW HA uptake and metabolism in EB3.

Poster Board Number: 2298

MOUSE EMBRYONIC STEM CELL-ENGINEERED TISSUE SHEETS WITH DEFINED CARDIAC CELL POPULATIONS AMELIORATE FUNCTION AFTER MYOCARDIAL INFARCTION

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BACKGROUNDS: Embryonic stem (ES)/ induced pluripotent stem (iPS) cells hold promise for cardiac regeneration therapies. Previously, we established a systematic cardiovascular cell differentiation system from mouse ES/iPS cells (Nature, 2000; Circulation 2008). In this system, cardiomyocytes (CMs), endothelial cells (ECs) and mural cells (MCs) can be induced from common progenitor, Flk1+ cells. In this study, combining our system and cell sheet technology using a temperature-responsive culture surface, we set out to reconstitute cardiac tissue by assembling ES/iPS cell-derived CMs/ECs/MCs, and examined the effect of the cardiac tissue sheet transplantation to myocardial infarction (MI) model. METHODS & RESULTS: Three cardiovascular cell types (CMs/ECs/MCs) were induced from mouse ES/iPS cells, purified by cell sorting, and plated on temperature-responsive culture dishes. Whereas pure CMs failed to efficiently form a sheet structure, CM/MC mixture successfully formed pulsatile cell sheets, suggesting a cell-integrative function of MCs. We generated cardiac cell sheets consisting of CMs (43.4±6.2% of total cells), ECs (3.3±2.6%), and MCs (53.3±8.2%). Multichannel extracellular potential analysis revealed that the cardiac tissue sheets hold unidirectional and regular electrical conduction, without ectopic foci (MED 64 system). Tri-layered ES cell-derived cardiac sheets were transplanted to a MI model of athymic rat heart one week after coronary ligation. Four weeks after the transplantation, echocardiogram showed a significant improvement of systolic function of left ventricle (LV) (fractional shortening: 36.2±7.6% vs 22.4±8.0, $p<0.01$, n=9) and a decrease in akinetic area in transplantation group. LV pressure-volume loop studies with catheter examination revealed higher values of end-systolic elastance compared to sham control (15.4±1.5 vs 8.8±2.2 mmHg/μl, $p<0.001$, n=8). Sirius red staining showed reduction of the infarct wall thinning (average thickness: 973±178 vs 705±192μm, $p<0.001$, n=25) and fibrotic area (MI length / total length: 24.8±7.4 vs 35.3±12.7%, $p<0.001$, n=25), indicating the attenuation of LV remodeling. Promotion of neovascularization in transplantation group was observed by von Willebrand factor staining. CONCLUSIONS: We succeeded in generating novel tissue sheets with defined cardiac cells from ES cells. Transplantation of cardiac tissue sheets significantly ameliorates cardiac function after MI. ES/iPS cell-engineered cardiac tissue may prove to be a promising strategy for efficient cardiac regeneration.

Poster Board Number: 2300

THE EXPANSION AND CARDIAC DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS IN THREE-DIMENSIONAL BIOREACTOR SYSTEM

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Although embryonic stem (ES) cells are known to differentiate into functional cardiomyocytes *in vitro*, current methods for inducing cardiac differentiation such as hanging drop might not be suitable for large scale cultivation to get enough amounts of cardiomyocytes for cardiac regeneration therapy. In this study, we show the established cultivation methods for expansion and cardiac differentiation of mouse ES cells using three-dimensional bioreactor system. To evaluate the cardiac differentiation efficiency, we used EMG7 mouse ES cells that expressed GFP under control of α-MHC promoter. We developed a robust and scalable bioprocess that allows direct embryoid body (EB) formation in a fully controlled, stirred 100 ml bioreactor with a pitched-blade-impeller following inoculation with a single cell suspension of mouse ES cells. Appropriate stirring conditions and dissolved oxygen resulted in the generation of high-density suspension cultures containing 7.0X106 cells/mL and 1,000 EBs/mL after 10 days of differentiation. Approximately 40% of the EBs in this process contained GFP positive cells and 10% of the EBs vigorously contracted, indicating robust cardiomyogenic induction. FACS analysis revealed that about 3% of cells expressed GFP, suggesting that bioreactor system might enable to expand ES-derived EBs that also contain cardiomyocytes. Next we tried to purify cardiomyocytes using mouse ES cells that expressed neomycin resistant gene under the control of α-MHC promoter. After 10 days in suspension culture with differentiation condition, EBs were treated with neomycin until 18 days. Almost all of remained cells after neomycin selection showed spontaneous beating and immunocytochemical analysis revealed that 99% of cells were positive for myosin heavy chain, 97% of cells were positive for sarcomeric α-actinin and 85% of cells were positive for cardiac troponin T, suggesting that drug-based purification system might useful in suspension culture. Furthermore, confocal microscopic analysis revealed that differentiated cardiomyocytes showed the fine striated pattern and some cells possessed double or triple nuclei, suggesting that suspension culture might enable to promote cardiomyocytes maturation. These findings suggest that optimized three-dimensional bioreactor cultivation might enable us to expand ES cells to get enough amounts of cardiomyocytes for cardiac regeneration therapy.

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Poster Board Number: 2302

GRAPHITE OXIDE NANOPARTICLES WITH DIAMETER GREATER THAN 20 NM IS BIOCOMPATIBLE TO MOUSE EMBRYONIC STEM CELLS AND CAN BE USED IN A TISSUE ENGINEERING SYSTEM

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Embryonic stem cells (ESCs) possess self-renewal property and robust differentiation potential. The pre-clinical transplantation of ESCs has demonstrated the ability to regenerate functional tissues. However, the clinical implementation of ESC therapy is hindered by limited engraftment and cell survival following transplantation, greatly compromising their restorative potential. In order to address this issue, a three-dimensional collagen hydrogel-based tissue engineering system has been designed to provide structural support and enhance cell engraftment following delivery. This platform can be integrated with graphite oxide nanoparticles (GO). The GO in their readily synthesized form are water-soluble single-layered sheets ranging in size from 3 to 600 nm in diameter. They allow electrical insulation, remain highly economical, and provide easy fabrication. With proper functionalization, hydrophobic biomolecules can be loaded onto the surface through pi-pi stacking, allowing controlled delivery and gradual release. Our long term goal is to apply tissue engineering in nanographite oxide technology to facilitate robust stem cell engraftment and survival. We propose to cross-link the GO to collagen fibers and anti-apoptotic molecule, neutrophorin 4 (NT4), and use this platform in conjunction with the delivery of ESC. The objective of this pilot study is to first optimize the size range of GO that is biocompatible on mouse ESCs followed by assessment of the effects of NT4 on ESCs. Mouse ESC line E14 was transduced with a reporter gene over-expressing luciferase. In the first study, GO was synthesized using a modified Hummers' method with varying levels of sonication and centrifugation to control the size of GO particles. We covalently conjugated branched poly(ethylene glycol) to the GO in order to impart biocompatibility. The mESCs were seeded at 25,000 cells/cm2, and GO particles of three size ranges (small: d=3-20 nm; medium: d=20-75; large: d=125-700) were dissolved in media at 0.005 mg/ml (low) or 0.01 mg/ml (high). For 7 days, control group received no GO and experimental groups received media containing GO of different size ranges and concentrations, resulting in a total of six groups: Small (size)/Low (concentration), Small/High, Medium/Low, Medium/High, Large/Low, Large/High. In the second study, mESCs receiving 0 or 10 ng/ml of mouse NT4 was maintained in hypoxia (5% CO2, 5% O2) to assess its pro-survival effect in an environment simulating ischemia over 72 hours of culture. Cell growth and phenotypic responses were evaluated. Atomic Force Microscopy and Dynamic Light Scattering of as-synthesized GO particles clearly showed three different size ranges. After 24 hours of culture, cellular uptake of GO particles was found in all groups. All cells grew over time while cells receiving small GO showed less proliferation and more cell death. 0.01 mg/ml of GO decreased cell growth compared to 0.005 mg/ml. Furthermore, small GO significantly decreased luciferase expression compared to all other sizes. No difference was found in the expressions of the pluripotency genes. Cells receiving NT4 showed higher cell growth, less cell death, and higher luciferase activity. In conclusion, GO particles larger than 20 nm in 0.005 mg/ml are biocompatible and can be safely used for mESCs; and NT4 is able to promote cell survival and growth under ischemic condition. Future study will focus on cross-linking GO to NT4 to control the release in our tissue engineering system.

Poster Board Number: 2304

MOUSE EMBRYONIC STEM CELLS ENGINEERED BONE AND CARTILAGE WITHIN A NOVEL PERFUSION BIOMIMETIC BIOREACTOR

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Introduction: Research in bone and cartilage engineering for regenerative medicine approaches has expanded over the last decade; however, few methods have been clinically translated. Partially because most engineered constructs lack anatomical and mechanical characteristics of native tissues, and can be resorbed after implantation. In this study, we have generated an engineered bone and cartilage construct (EBCC) within a biomimetic bioreactor. This work has leveraged from our recent studies, which demonstrated collagen 1 matrix polymerised with β-glycerol phosphate (BGP) as a potent inducer for osteogenesis. Cultivation of mouse and human embryonic stem cells (ESCs) with the matrix *in vitro* triggered osteogenesis. Furthermore, a switch to chondrogenesis can be obtained when chondroitin sulphate is supplemented. This data coupled with the fact that no teratomas or tumors were observed when the construct was transplanted into SCID mice sub-cutaneously is promising. However, prolonged maintenance of cell viability, media changes and application of mechanical stimulants that drives further maturation of chondrocytes in a petri-dish limits the application of this engineered construct. Methodologies: A perfusion bioreactor supported with a peristaltic pump was designed to provide a continual flow of media to the EBCC at constant flow rate. This design allows alternation of media compositions in real-time, based on the needs of the EBCC at each stage of differentiation. An isolated BGP polymerized collagen 1 platform was fabricated within the bioreactor which supports ESC differentiation. Oxygen and nutrients are delivered to the cells through passive diffusion across an agarose intermediate to mimic physiologic conditions. Nutrient and oxygen gradients developed within the bioreactor are based on the design of bioreactor and seeding density of ESCs. This gradient is exploited (with media supplementation) to promote both osteogenesis and chondrogenesis resulting in EBCC formation. Furthermore, the materials used to assemble the bioreactor are amenable to direct mechanical stimulation (bending and compression) and is applied to the developing EBCC to enhance differentiation. Results: The EBCC is viable within the bioreactor for at least 30 days post inoculation, with cellular remodeling observed in constructs cultivated with active perfusion. Quantitative PCR (qPCR) analyses demonstrates a significant decrease in the pluripotent marker, OCT-4, after 30 days, with coinciding increases in osteogenic and chondrogenic markers. Conclusion: We have shown the capability of our bioreactor to maintain long term cultivation. The composition of media could be adjusted according to the needs of the cells at different stage of development. We will be applying mechanical stimulants on the engineered construct to drive further maturation, to achieve constructs with similar anatomical and mechanical characteristics as native tissues. Further, the collagen-based platform will be segregated based on exposure to oxygen determined through a fiber-optic probe for a more comprehensive study.

Poster Board Number: 2306

FORMULATIONS FOR CONCURRENT PROTEIN DELIVERY FROM MICROPARTICLES AS A METHOD FOR INDUCTION OF TISSUE REGENERATION

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INTRODUCTION: The controlled release of proteins from microparticles (MP) is a key strategy in regenerative medicine. The protein may act as an

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angiogenic factor or a growth factor to stimulate tissue formation once they have been release from MP *in vivo* or *in vitro*. To achieve functional tissue regeneration multiple protein delivery might be needed depending of the target tissue. On the other hand, the proteins must be presented in an active form within the therapeutic window for sufficient time to complete the tissue formation process. In this abstract we present an approach to pre-program protein release from poly(lactic acid-co-glycolic acid) (PLGA) MPs. This approach uses a surfactant to control protein release as a function of water penetration. MPs may or may not be loaded with cells to help regeneration of the damaged tissue. This information would be further used for concurrent or sequential delivery of growth factors that is shown to play a crucial rule in regenerative medicine. EXPERIMENTAL METHODS: poly(ethylene glycol) with (Mw 1000), DL-lactide, glycolide, stannous octoate were used to synthesise PLGA-PEG1000-PLGA triblock copolymer(TB IV) following a method described by Zentner et al. . Triblock is characterised using 1H-NMR and GPC. Fluorescein isothiocyanate (1 mole) was dissolved in borate buffer (pH 8.3 to 9) and stirred at room temperature for 48 hrs followed by addition of lysozyme (1 mole) to the FITC solution and stirred for another 48 hrs. Lyso-FITC solution was dialysed against borate buffer for 72 hrs to exclude excessive FITC. Lyso-FITC solution was then freeze-dried and degree of labelling was calculated. HSA/Lyso-FITC was encapsulated in MPs using water-in-oil-in-water emulsion method. Briefly, Lyso-FITC and HSA were mixed in 1:9 ratios and dissolved in water. PLGA and PLGA-PEG1000-PLGA triblock were dissolved in DCM in a 7:3 or 9:1 (w/w) ratio. After complete dissolution, protein was added to the polymer solution and homogenised for 2 min at 4000 rpm using a Homogeniser. This was followed by addition of the protein-polymer mix to a 0.3% PVA bath and homogenised for second emulsion for 2 min at 2000 rpm. MPs were left to harden in a 0.3% PVA bath for 4 hrs stirring at 300 rpm. To investigate the Lyso-FITC release from each of formulations 50 mg pf MPs were added to a vial and 1.5 ml PBS was added to the top. Samples were collected as certain time points. Release of Lyso-FITC from HSA/Lyso-FITC loaded MPs was measured by measuring fluorescent intensity. RESULTS AND DISCUSSION: The spectra obtained (figure 1) were similar to spectra reported previously. To obtain the number average molecular weight, the peaks at 5.20 ppm (CH of LA), 4.80 ppm (CH2 of GA), 3.65 ppm (CH2 of ethylene glycol), and 1.55 ppm (CH3 of LA) were used. The polydispersity indexes of the triblocks and composition ratios of LA/GA were determined via 1H-NMR and GPC. The release profile from six different formulations is shown in figure 2. In search for formulations that release protein in certain period of time, foundation polymers that contain different amount of glycolide was used. This formulation might be used for drugs which their presence is required for short period of time and in high dosage such as VEGF and PDGF delivery. CONCLUSION: Based on these data, the addition of the surfactant to the particles can be used to modify the release kinetics of a model protein. Formulations introduce here might be used for concurrent or sequential delivery of multiple drug proteins in a pre-programmed manner.

Poster Board Number: 2308

SCREENING FOR CELL CULTURE SUBSTRATE TOPOGRAPHY THAT ENHANCE DIFFERENTIATION OF NEURAL PROGENITOR CELLS INTO MATURE NEURONS

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Replenishing the nervous system with stem cell derived functional neurons may be a viable treatment for neurological diseases such as Parkinson's disease. However, a major bottleneck lies in the current inability to direct the differentiation of stem and progenitor cells quickly and efficiently to the neuronal subtypes of interest, and this has to be overcome. Our group had shown that nano-scale extracellular matrix topography can influence cell fate decisions of stem and progenitors cells and enhance differentiation. For example, human mesenchymal stem cells (hMSCs) can be induced to differentiate into neurons by culturing cells on nano-gratings on poly(dimethylsiloxan) (PDMS) substrate. Based on our observation, we hypothesize that topographies with various geometries and sizes can have different and specific effects on the stem cell fates and selection of the appropriate topographical cues will enhance the neuronal differentiation into different sub-type of neurons. Here, we conducted a screen for cell culture substrate topographies that are able to direct differentiation of neural progenitor cells (NPCs) isolated from the hippocampus of postnatal day 5 old mouse into specific neuronal subtypes of interest. Interestingly, we were able to find anisotopic patterns that preferentially differentiate multipotent NPCs into neurons over the astrocytic and oligodendrocytic lineages. We also found that certain topographical parameters such as the microscale pillars enhance the differentiation of NPCs into forebrain neurons. In conclusion, we have gained important insights from this screen that could be adapted for a more efficient derivation of mature neurons for neurological repair.

Poster Board Number: 2310

QUANTITATIVE CELLULAR AND MOLECULAR SCREENING STRATEGIES FOR MYOCARDIAL INFARCTION REVEAL A POTENTIAL CARDIAC REGENERATIVE THERAPY

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Myocardial infarction (MI) is the leading cause of congestive heart failure and death in the world. Coronary occlusion and the resultant myocardial ischemia rapidly results in myocardial necrosis followed by fibrotic scar formation. Clinical trials for cell transplantation to treat MI have been conducted mostly using bone marrow cells wherein modest (at best) improvements in cardiac function have been attributed to paracrine effects. Next generation strategies to treat MI include transplanting cardiomyocytes (CM) or cardiac progenitors (CP) (either on their own or as part of *ex vivo* engineered cardiac tissue), and endogenous cardiac tissue repair using molecules or genes. Developing the most suitable conditions to bring forward for clinical development is made signicnaflty more complicated by the lack of efficient and predictive strategies to screen the large numbers of parameters that may impact the success of these strategies. We are using cardiac tissue models, engineered heart tissue (EHT), and quantitative molecular and electrophysiological analyses, as a test-bed to screen regenerative conditions for their potential to functionally integrate with the host tissue. In the area of cell transplantation, EHT was prepared by seeding neonatal rat CM onto porous collagen scaffolds and subjecting the resulting constructs to electrical field stimulation (biphasic pulses, 1ms duration per phase, 1Hz) for 5 days as described. This platform was validated by injection of contractile mouse neonatal (n) CM, non-contractile cardiac FB and embryonic stem cells (ESC). As expected, FB injection interfered with electrical signal propagation, ESC formed teratoma-like structures uninfluenced in their differentiation trajectory by the *in vitro* cardiac microenvironment, and injected nCM improved tissue function. Interestingly ESC-derived CP (Flk+/PDGFRα+ cells), but not ESC-derived CM (generated using MHCα-driven antibiotic selection) appeared able to appropriately mature and integrate into EHT, enhancing the amplitude of tissue contraction, propagating electrical signals, supporting the viability of the host EHT and exhibiting Ca2+ transients in synchrony with the host EHT. Recently, using the EHT model system, we began to quantitatively test the impact of *in situ* fibroblast-to-CM reprogramming using regulated expression of the cardiac transcription factors MEF2c, Gata4 and Tbx5. This reprograming strategy resulted in about 15 % cTnT + cells

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with upregulation of other cardiac genes such as Mesp1. In addition, to accelerate the screening efficiency in reprogramming, we engaged a self-organized micro tissue platform using mouse embryonic FB and CM. These cardiac model systems are powerful tools to rapidly and qualitatively screen cellular and molecular based strategies to regenerate cardiac tissue in MI-like scenarios.

Poster Board Number: 2312

EFFECTS OF THE DESIGNED SELF ASSEMBLING PEPTIDE NANOFIBERS ON DIFFERENTIATION OF MARROW-DERIVED CARDIAC STEM CELLS AND CARDIAC REPAIR

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Stem cell therapy is a promising therapeutic strategy for treating myocardial infarction. However, the efficacy of the procedure remains hampered by a low differentiation efficiency of the engraftment cells toward cardiomyocytes. In recent years, synthesis and application of injectable nanomaterials have opened a brand-new research field for therapy of stem cell transplantation. In this study, a novel self-assembly peptide possessing functional motifs to attach cells and bind BMP-2 was used as carrier of stem cells and scaffold with biological and functional properties in transplantation. The functional self-assembly peptide can assemble spontaneously into well-ordered nanofiber scaffolds. Marrow-derived cardiac stem cells (MCSCs) were isolated from rat bone marrow by single-cell clone culture and RT-PCR analysis. The cells seeded in the self-assembly peptides spread well and attached tightly on the nanofiber scaffolds. At 3 and 7 days after incubation in the self-assembly peptide nanofiber (SPNF) scaffolds, survival of the cells were determined with AO/EB double staining. There were more survived cells in SPNF group than that in control group. After treatment with oxygen and glucose deprivation, the apoptotic and necrotic cells were less significantly in SPNF group than that in control group. After induction with BMP-2 for two weeks, the cells in SPNF showed a tendency to parallel in arrangement and expressed cardiac-specific troponin T (cTnT). After induction for four weeks, the cells displayed myotube-like structures, cTnT expression of the cells was increased. SPNFs have been degraded partly. After injection into rat myocardial infarction models, immunogenicity of the peptides was very low. Most SPNFs have been degraded at four week after injection, only some SPNF fragments were observed between cardiomyocytes. The echocardiographic examination showed that EF and FS increased significantly in MCSC-SPNF group compared with MCSC and SPNF alone groups. Quantitative analysis demonstrated that collagen volume fraction in MCSC-SPNF group was smaller than that in MCSC and SPNF alone groups. Fluorescence *in situ* hybridization indicated that there were more Y chromosome-positive cells in MCSC-SPNF group at three week after transplantation. Most Y chromosome-positive cells expressed cTnT and connexin 43. Connexin 43 was located between Y chromosome positive-cells and recipient cardiomyocytes. These results show that the self-assembly peptides represent a good biocompatibility for MCSC attachment and growth. Cytokines released slowly from SPNF induce MCSCs to differentiate into cardiomyocytes. Transplantation of stem cells in SPNF is a useful technique for repairing the myocardium and improving cardiac functions.

Poster Board Number: 2314

HYPOXIC CONDITIONS HAVE DIFFERENTIAL EFFECTS ON COLONY FORMATION, PROLIFERATION AND *IN VITRO* ANGIOGENESIS OF ENDOTHELIAL PROGENITORS.

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Introduction: Bioengineered tissue requires a vascular network in order to access oxygen and nutrients. Cord blood derived endothelial progenitor cells (CEPC) and umbilical vein endothelial cells (HUVEC) are widely used to facilitate neo-vascularization (1-2). However, CEPCs or HUVECs delivered *in vivo* face hypoxic conditions, and it is not completely understood how and to what extent hypoxic conditions affect angiogenesis resulting from implantation of these cells. In this study, we examined the effect of hypoxic conditions on the viability, clonogenic ability, proliferation and angiogenesis potential of both CEPCs and HUVECs . Materials and Methods: Colony forming assay: HUVECs and CEPCs were sorted using a BD FACSAria II and dispersed as single cells into individual wells of a 96-well plate. Each well was pre-filled with 200µL of EGM10 medium. The cells were cultured in normoxic and hypoxic conditions (0.1%, 3%, or 20% O2) for seven days and then stained with Crystal Violet. Colonies were then counted, and the number of cells within each colony was determined. Angiogenesis potential (Dynamic Tube Forming Assay): HUVECs and CEPCs were seeded on 96-well plates containing Matrigel (BD Biosciences) at a seeding density of 5000 cells per well. Cells were then cultured in normoxic and hypoxic conditions for eight days. Images were taken each day at 40X with a Zeiss Axiovert microscope and analyzed using the Image J plug-in NeuronJ to quantify tube length. MTS cell proliferation assay vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF) was also added to some samples of HUVECs and CEPCs. The MTS assay was then performed at daily time points in normoxic and hypoxic conditions to assay cellular proliferation. Results: The colony forming assay showed that about 50% of the wells developed colonies, and there appeared to be no difference in the colony forming ability of these cells at different oxygen conditions. However, at high oxygen levels, there was an increase in the number of larger colonies for both cell types. The dynamic tube forming assay demonstrated that in low oxygen conditions (0.1% and 3%), HUVECs and CEPCs were able to form and maintain complex tube networks over 7 days, while in high oxygen conditions (20%), the tube networks disintegrated. The proliferation assay showed that the proliferation of both cell types slowed under hypoxic conditions. However, compared to the control culture conditions (EBM2 culture medium alone), 10ng/mL bFGF or 50ng/mL of VEGF in the media were able to rescue the cells, and the cell number increased under hypoxic conditions when these growth factors were present. Discussion and Conclusion: In this study, we observed that hypoxic conditions affect the proliferative ability and angiogenic potential of CEPCs and HUVECs, but colony forming ability was not altered. These findings demonstrate that hypoxic conditions have separate effects on colony forming, proliferation and tube forming ability, and thus, these may represent three independent biological responses to hypoxic stress. Overall, this study indicates that low oxygen concentrations, existing at the center of implanted bioengineered tissues, have a significant effect on the activity of endothelial cells, this may affect their ability to contribute to tissue neovascularization.

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IN SITU CARDIOMYOGENIC DIFFERENTIATION OF IMPLANTED BONE MARROW MONONUCLEAR CELLS BY HEPARIN CONJUGATED PLGA NANOSPHERES WITH TRANSFORMING GROWTH FACTOR-BETA1

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Bone marrow mononuclear cells (BMMNCs) can be used to treat patients with myocardial infarction, since BMMNCs can differentiate *in vitro* toward cardiomyogenic lineages when treated with transforming growth factor-β1 (TGF-β1). However, the *in vitro* cardiomyogenic differentiation culture process is costly and laborious, and the patients should wait during the culture period. In this study, we hypothesize that BMMNCs implanted in cardiomyogenically undifferentiated state to myocardial infarction site would differentiate cardiomyogenically *in situ* when exogenous TGF-β1 is delivered to the cell implantation site. Heparin-conjugated poly(lactic-co-glycolic acid) nanospheres (HCPNs) suspended in fibrin gel were used as a TGF-β1 delivery system. BMMNCs were labeled with a green fluorescent dye (PKH-67) and implanted into the infarction border zone of rat myocardium using fibrin gel containing HCPNs and TGF-β1. BMMNC implantation using fibrin gel and HCPNs without TGF-β1 served as a control. Four weeks after implantation, the expression of cardiomyogenic marker proteins by the implanted BMMNCs was dramatically greater in the TGF-β1 delivery group than in the control group. This method can significantly improve the stem cell therapy technology for myocardial regeneration, since it can remove *in vitro* cell culture step for cardiomyogenic differentiation prior to cell implantation.

Poster Board Number: 2318

DEVELOPING A MINIMALLY-INVASIVE INJECTABLE HYDROGEL SYSTEM FOR CO-DELIVERY OF ADULT RAT NEURAL STEM CELLS AND PLATELET-DERIVED GROWTH FACTOR-AA

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Stem cell transplantation presents a promising strategy for the treatment of a variety of CNS disorders. However, it is broadly recognized that there is poor survival of transplanted cells, even in the "immunoprivileged" CNS. This is primarily due to the notoriously inhibitory environment that exists after injury. To promote differentiation and enhance survival of transplanted neural stem cells (NSCs), various factors have been delivered via catheter and mini-pump to the intrathecal space including Epidermal growth factor (EGF), Fibroblast growth factor 2 (FGF2), and Platelet-derived growth factor-AA (PDGF-AA) (1). Yet the cell delivery/minipump method is invasive and has risks of infection. We have designed a minimally-invasive, injectable and biodegradable hydrogel blend of hyaluronan and methylcellulose (HAMC). HAMC has been shown to promote survival of retinal stem/progenitor cells (2). Here we describe the development and *in vitro* optimization of HAMC to enhance survival of NSCs by incorporation of cell adhesive peptides and growth factors. HAMC allows uniform cell distribution. Functionalization of methylcellulose to incorporate reactive carboxylic groups allows conjugation of cell adhesive peptides to enhance cell adhesion. PDGF-AA treatment enhances survival and promotes differentiation of NSCs primarily into oligodendrocytes (70.9%), with very few astrocytes (0.9%) and negligible neurons (0.01%). In ongoing studies we are examining co-delivery of PDGF-AA and cell adhesive peptides in HAMC for enhanced cell survival of NSCs. The long-term goal is to test the efficacy of this hydrogel system in enhancing *in vivo* survival of NSCs after transplantation to the injured spinal cord.

Poster Board Number: 2320

BLADDER STRAIN INDUCED MICROENVIRONMENT ATTENUATES SMOOTH MUSCLE DIFFERENTIATION OF ADULT RAT SKIN DERIVED PRECURSOR CELLS: IMPLICATIONS FOR TISSUE REGENERATION

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Smooth muscle cell containing organs such as bladder, heart and blood vessels are targeted by a variety of pathological conditions necessitating surgery or organ replacement. Currently, tissue engineering approaches are hampered by lack of functional smooth muscle cells. Multipotent Skin derived progenitor cells (SKPs) can easily be isolated from adult skin and can be differentiated into a variety of cell lineages. Here we describe the opposing effects of FBS and the bladder microenvironment on induction of smooth muscle cell differentiation in SKPs. SKPs cultured in presence of FBS strongly upregulate expression of smooth muscle marker such as smooth muscle actin, calponin, myocardin and myosin heavy chain compared to SKPs cultured in EGF/FGF containing medium as demonstrated by real time PCR and IF. FBS treated SKPs organize smooth muscle actin into cytoskeleton actin cables and contract collagen I gels, two characteristics of functional smooth muscle cells (SMC). *In vivo*, bladder strain induces microenvironmental changes lead to de-differentiation of fully differentiated bladder SMC. To investigate how this strain induced bladder microenvironment modifies SMC differentiation of SKPs, we differentiated SKPs in presence of bladder organoids or conditioned medium derived from stretched or relaxed bladders. Diffusible factors released from stretched bladders decreased SMC differentiation of SKPs. Inhibition of mTOR signaling using Rapamycin treatment, previously observed to promote SMC differentiation of fully differentiated bladder SMC *in vivo*, revealed here that FBS induced SMC differentiation of SKPs requires mTOR signaling. These results suggest that SKPs could be used as a source for SMCs in regenerative strategies for hollow organs such as the bladder. However, it is also apparent that organ-specific microenvironmental contexts play a significant role in modulating or even inhibiting stem cell phenotype, possibly explaining the variable or less than exuberant differentiation of stem cell constructs in *in vivo* settings. These observations must be considered in drafting any overall regeneration strategy.

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TOTIPOTENCY/EARLY EMBRYO CELLS

Poster Board Number: 2322

COMPARING HUMAN TROPHECTODERM WITH EMBRYONIC STEM CELLS: DELINEATING THE FIRST DEVELOPMENTAL BIFURCATION

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The first week of human development is characterized by the differentiation of the early embryo into the inner cell mass (ICM) and trophectoderm (TE). Whereas the ICM, through its derivative, human embryonic stem cells (hESC), has been largely reported, by contrast, the human TE at blastocyst stage is still unelucidated. TE is of prime importance since TE not only plays a major role in the shaping of the blastocyst stage but also latter develop into placenta that will provide the nutrient support for further embryonic development. The aim of the present study is (i) to establish the human TE transcriptome by whole genome expression profiling and (ii) to compare with hESC transcriptome. Five TE samples were mechanically separated from fresh embryos issued during an *in vitro* fertilization program. The human embryos were donated to research after informed consent from the couples. This embryo research has received an IRB agreement. Each TE was individually analyzed by whole genome U133P Affymetrix (Santa Clara, CA) oligonucleotides microarrays. Gene enrichment is analyzed with Fatigo (BABELOMICS) and statistical analysis was carried out with SAM (Stanford, CA). Gene expression profile was illustrated by Amazonia! (http://amazonia.transcriptome.eu/). Using SAM, we delineated a «TE signature» comprising 975 probesets (PS) overexpressed in TE comparing to hESC. Through a gene enrichment analysis of TE signature, TE showed significantly increased protein synthesis activity and decreased extracellular molecules expression. 19 genes in TE signature display unique profile in TE. Comparing with hESC, SAM result showed 16 common transcription factors (TF) in placenta and TE. With these TF, a network was built up using Ingenuity System. All TF in this network was shown simultaneously up-regulated in an *in vitro* model established by inducing hESC and hiPS cell lines into TE differentiation though BMP4 treatment. This network deserves further studies although a few of them have the known functions in placenta development, TE differentiation or maintenance. Our results show for the first time the transcriptome difference between TE and pluripotent stem cells (PSC) at whole genome scale. TE was shown having very differed expression profile to PSC, just few days after the differentiation from ICM. Expression of TE-specific genes corresponds to its special functions. A persisting TF network was built up and may play key roles in the first bifurcation of cell fate in whole human development. (2135 characters)

Poster Board Number: 2324

ROLES OF KLF5 IN ESC PLURIPOTENCY AND EARLY EMBRYONIC DEVELOPMENT

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ES (Embryonic Stem) cells are derived from the blastocyst and have the potential to give rise to derivatives of each germ layer. Induced pluripotent stem (iPS) cells can be derived from lineage-restricted cells, such as fibroblasts, by forced expression of specific transcription factors. Although recent studies indicate that Krüppel-like factors (Klfs) are essential for both maintenance of ES cell self-renewal and reprogramming of somatic cells into a pluripotent state, the molecular mechanism of these processes remains unknown. Thus, understanding the molecular mechanism of ES cell self-renewal by Klfs would be important for the efficient generation of patient-specific pluripotent stem cells and for the development of regenerative medicine. Although we have showed that Klf5 is indispensible for blastocyst development and the derivation of mouse ESCs from the inner cell mass (ICM), the molecular mechanism underlying these functions remains unknown. To understand the mechanism, we first addressed the precise phenotype of Klf5 KO mouse embryos. By investigating the BrdU incorporation activity and the pluripotency-related and lineage-marker expressions in Klf5 knock-out embryos, we show that Klf5 is indispensible for normal cell-cycle progression of early stage of mouse embryos, which may be consistent with the previous our finding that Klf5 is an essential regulator for the cell-cycle process of mouse ESCs. We also show that comparable Cdx2 and Oct3/4 gene expressions are seen in Klf5 KO embryos at morula stage. While Nanog and Cdx2 gene expression are decreased significantly in Klf5 KO embryo at blastocyst stage, the development of Klf5 KO embryos is severely retarded, suggesting that the reduced expression of Nanog may be due to the retardation of development. Members of the Klf family such as Klf2, 4 and 5 are expressed abundantly in the ICM and down-regulated as the cells differentiate into epiblasts, which suggest that the down-regulation of Klf2, 4 and 5 is required for correct differentiation into epiblasts. By using over-expressing ESCs for Klf family members, we show that Klf family members have overlapped and non-overlapped functions.

Poster Board Number: 2326

BONE MARROW-DERIVED RAT MULTIPOTENT ADULT PROGENITOR CELLS ARISE FROM PROLONGED *IN VITRO* CULTURE AND ARE SIMILAR TO BLASTOCYST-DERIVED EXTRAEMBRYONIC ENDODERM PRECURSOR CELLS

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Debeb et al. (2009) recently described the isolation of extraembryonic endoderm precursor (Xen-P) cell lines from rat blastocysts, which contribute to visceral and parietal endoderm and to trophoblast following morula

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aggregation, and form yolk sac like tumors. Here, we demonstrate that the rat (r) multipotent adult progenitor cell (MAPC) lines, described by Breyer et al (2006) and Ulloa et al (2007), exhibit salient Xen-P-like characteristics. Cells with Xen-P-like features (expression of SSEA1, CD31, Oct4, Gata4, Gata6, Sox17, Sox7, and Foxa2) emerged after 8-12 weeks of culture of rat bone marrow (BM) in MAPC culture conditions. However, we were unable to retrospectively identify similar cells in uncultured or short time cultured rat BM cells by FACS, qRT-PCR and Oct4 immunohistochemistry. Unlike BM-derived MAPC, the new Xen-P-like cell lines, we now report, were isolated from rat blastocysts in 2-8 days, in rMAPC medium. Moreover, established Xen-P clones, grown under rMAPC conditions (5% O2, 2% FBS, LIF, PDGF, EGF on fibronectin at ±300 cell/cm2) grew dispersed and showed a uniform morphology, as is typically seen for rMAPC, and expressed nearly homogenously SSEA1, CD31, Oct4, Gata4, Gata6, Sox17, Sox7 and Foxa2. When rMAPC lines were cultured under Xen-P conditions (20% O2, 15% FBS and LIF on rat embryonic fibroblasts) they acquired the typical morphology of Xen-P colonies: round cells at the border expressing SSEA1, Oct4 and Gata4 protein, whereas the more flattened cells in the center of the colonies were low or negative for SSEA1 and Oct4 but remained strongly Gata4 positive. When comparing cells isolated under rMAPC conditions from BM, the new blastocyst-derived cell lines obtained under MAPC conditions, and traditional Xen-P cell lines cultured under rMAPC conditions, we did not observe significant differences in expression of cell surface CD31 and SSEA1, nor in their expressed gene profile. Consistent with the high levels of Oct4 expression, the Oct4 promoter was hypomethylated in cells isolated under MAPC conditions from either BM or blastocysts, and growth factor requirements were almost identical. In particular, LIF withdrawal resulted in complete growth arrest of both cell types after two passages, downregulation of Oct4 and significant induction of the visceral/parietal endoderm transcripts Hnf4a and Afp and the visceral endoderm transcript Tmprss2, suggesting default differentiation to visceral endoderm. *In vitro*, the differentiation ability of cells isolated under rMAPC conditions from blastocysts to smooth muscle-like, hepatocyte-like and neuroprogenitor-like cells was similar to that of cells isolated from BM under rMAPC conditions. *In vivo*, cells isolated under rMAPC conditions from either BM or blastocysts, as was previously shown for Xen-P cells, could contribute to the extraembryonic endoderm of the developing embryo after morula aggregation, and generated yolk-sac like tumors when transplanted subcutaneously or under the kidney capsule of immunocompromised mice. In conclusion, these studies provide a dramatically improved method for isolation and culture of almost homogeneous Xen-P cell populations, and demonstrate that rat MAPC are most likely a culture-induced equivalent of Xen-P cells.

Poster Board Number: 2328

NANOG REGULATES ENDODERM FORMATION THROUGH THE MXTX2-NODAL PATHWAY

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During early embryogenesis, the acquisition of pluripotency is dependent upon Nanog. In zebrafish, we identified the nanog ortholog, and found that its knockdown impaired endoderm formation. The expression of nodal genes that are required for endoderm induction was specifically lower in nanog morphants. And the endoderm defect could be rescued by restoring nodal, suggesting nanog regulates endoderm formation by activating nodal. Genome-wide transcriptional analysis revealed that nanog morphants fail to develop primitive endoderm. The primitive endoderm was shown to secrete nodal necessary for definitive endoderm induction. Recent studies have shown that Nanog null mouse embryos do not develop primitive endoderm, suggesting a conserved role of nanog in regulating primitive endoderm formation during vertebrate evolution. In an effort to define critical regulators of primitive endoderm, we examined the genes that were differentially regulated by nanog deficiency, and identified the homeobox gene, mxtx2. mxtx2 is one of the earliest genes expressed in primitive endoderm precursors, and its expression is absent in nanog morphants. Using morpholinos, we demonstrated that mxtx2 is required for primitive endoderm induction. By overexpression of mxtx2, we were able to restore the primitive endoderm and rescue the nodal and endoderm defects in nanog morphants. To evaluate if mxtx2 is a direct target of nanog, we developed a novel method in zebrafish to undertake ChIP-Seq analysis. Myc tagged nanog or mxtx2 was injected as mRNA into 1-cell embryos, and resulting embryos at early or late blastula stage were subjected to chromatin immunoprecipitation assays. The global binding analysis found that nanog directly activates mxtx2 and mxtx2 binds specifically to primitive endoderm genes. Our study discovered a novel nanog-mxtx2-nodal pathway, and established a role of nanog in regulating the formation of the primitive endoderm required for definitive endoderm induction.

HEMATOPOIETIC STEM CELLS

Poster Board Number: 2332

SUSPECTED INCOMPLETE REPROGRAMMING IN BLOOD DERIVED HUMAN INDUCED PLURIPOTENT STEM CELLS IS ADVANTAGEOUS FOR ADULT-TYPE ERYTHROCYTE GENERATION WITH GLOBIN SWITCHING

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It is known that "globin switching" during erythropoiesis is associated with the pathophysiology of sickle cell anemia, as well as an approach to ameliorating some hemoglobinopathies. Human pluripotent stem cells are attractive tools for studying the ontogeny of human erythropoiesis because they exhibit *in vitro* differentiation toward various erythrocytes with embryonic (ε), fetal (γ) or adult (β) globin genes, and they are amenable to gene manipulation in treating diseases. However, earlier studies showed that erythroid differentiation from human ESCs/iPSCs (hESCs/hiPSCs) stopped at fetal erythrocytes. In addition, successful differential reprogramming through somatic cell nuclear transfer and the use of iPSCs raised to the possibility that the poor yield of β-globin-expressing erythrocytes may result from incomplete genomic methylation or deregulated epigenetic modification. Recent studies using mouse iPSCs suggest the presence of "epigenetic memory" reflecting the tissue of origin. It has been also suspected that aberrant DNA methylation in iPSCs provides may lead to dominant differentiation to the original cell lineages, however in mouse iPSCs this tendency was observed in less than 10 passages. Mouse iPSC are known as a ground state-pluripotent stem cell enabling viable chimera mouse as a result of reset of DNA methylation from original cells. However, the quite huge distinction between mouse and human iPSCs may also raise the idea that hiPSCs derived from targeted cell lineage could be a promising source for specific cell therapy. In that context, we attempted to establish an *in vitro* differentiation culture system that would preferentially yield adult-type erythrocytes derived from hiPSCs and would enable the study of β-globin gene switching during erythrocyte ontogeny. We initially established iPSCs from human

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dermal fibroblasts (HDFs) (f-iPSCs) or cord blood-derived CD34+/CD45+ cells (b-iPSCs). Gene expression analyses showed comparable patterns of gene clustering in f-iPSCs and b-iPSCs, suggesting that hiPSCs retain some memories of original cells. We then compared the hematopoietic colony forming capacities of 6 f-iPSC clones and 10 b-iPSC clones using a previously established culture system. The b-iPSCs produced a higher number of mixed-lineage or BFU-E colonies than f-iPSCs (Mixed colonies: 261±39.4 vs. 36±13.8, p<0.01; BFU-E: 192±35.4 vs. 11.8±6.7 per 1x105 iPSCs, p<0.01). We selected two clones from each group to further analyze erythroid maturation and globin switching in erythrocytes generated from b-iPSCs and f-iPSCs. RT-PCR and immunochemical assays revealed limited differentiation by f-iPSC-derived erythrocytes (i.e., most were at the fetal stage), which is consistent with previous reports (Chang, Blood 2010), but b-iPSCs efficiently generated adult-type erythrocytes expressing β-globin (f-iPSCs, 27.6±15.8% vs. b-iPSCs, 79.9±11.7% β-globin+ p<0.01). Quantitative PCR analysis for known regulators of globin switching, such as BCL11A, KLF1, GATA1, and FOG1 shows no clearly difference between f-iPSCs and b-iPSCs. This dominant generation of adult-type erythrocytes is observed in human b-iPSCs independently of passage number, i.e., over 40 passages. These results suggest that incomplete reprogramming possibly by aberrant DNA methylation may promote preferential differentiation to original cell lineage using hiPSCs, contributing to stable supply of adult-type erythrocytes toward clinical applications.

Poster Board Number: 2334

FUNCTIONAL DENDRITIC CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS VIA HEMANGIOBLASTS

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Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) of the adaptive immune system. Upon encountering foreign cells, DCs proteolyze and present pathogenic or tumor-associated peptides to induce antigen-specific immune responses from both memory and naïve T cells as well as B cells, thereby protecting the body against viruses, bacteria, and cancer. Clinically, DCs may have potential utility in vaccine-based therapies against cancer and/or HIV due to their ability to stimulate specific immune responses from a patient's own adaptive immune cells. Several DC-based vaccine therapies are currently being tested in clinical trials using autologous and allogenic DC sources, yet these procedures are quite expensive to perform and must be prepared on an individual basis. Human embryonic stem cells (hESCs) offer an alternative cell source for the derivation of DCs. Since hundreds of disease-associated antigenic peptides have already been discovered and well-characterized, hESC-derived DCs could theoretically be used with such peptides in cost-effective, off-the-shelf DC vaccine therapies. To explore this possibility, we have used a hemangioblast differentiation system to generate DCs from multiple hESC lines including H1, H7, H9, and our own single-blastomere derived MA01 hESCs. Hemangioblasts (blasts) serve as the precursor to both hematopoietic and vascular cell lineages and our lab has shown that they can be generated in large scale quantities *in vitro*. With respect to hematopoietic differentiation, our lab has previously shown that blasts can efficiently differentiate into megakaryocytes /erythroid cells, thereby facilitating the *in vitro* production of mature red blood cells and functional megakaryocytes/platelets in large scale quantities. Here, we describe for the first time, the use of hESC-derived hemangioblasts for the production of functional myelomonocytic CD11c+ DCs. In comparison to previously published embryoid body based methods, our hemangioblast system offers comparable efficiency (2-5 DCs per starting hESC) in less time (20-24 versus 30-50 days) and can be performed in a serum- and feeder-free manner, which is critical for future clinical translation. Our blast-derived CD11c+ DCs display morphologically distinct dendritic processes and express characteristic DC surface markers such as CD209, HLA-ABC (class I), HLA-DR (class II), CD86, CD83 and CD45. They migrate in response to the chemoattractant MIP3β, take-up and proteolyze antigen, and can be induced to secrete the Th1-directed cytokine IL12p70. Importantly, blast-derived DCs can induce T cell proliferation in a mixed leukocyte reaction assay and can be used to generate antigen-specific T cell populations. Overall, this study further expands the utility of our hemangioblast differentiation system to include the production of myelomonocytic lineage cells and shows that large scale hemangioblast production can potentially be used for the development of future off-the-shelf DC-based vaccine therapies.

Poster Board Number: 2336

DIFFERENTIATION POTENTIAL OF CELL SPECIFIC HUMAN INDUCED PLURIPOTENT STEM CELLS INVERSELY CORRELATES WITH REPROGRAMMING EFFICIENCY

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In the present study, we sought to quantify the efficiency of induced pluripotent stem cell (iPSC) reprogramming and subsequent *in vitro* hemato-endothelial differentiation potential from different human primary cells. These starting cell populations include neonatal human dermal fibroblasts (NHDFs), CD34+ umbilical cord blood cells (UCBs), and dental pulp cells (DPCs). iPSCs were readily generated from all of these cell sources by lentiviral transduction of a polycistronic cassette harboring OCT4, SOX2, KLF4, and cMYC. Reprogramming efficiencies were quantified by scoring putative iPSC colonies present at 26 days post-transduction. UCBs generated iPSC colonies (UCB-iPSCs) at an estimated efficiency of 0.05%, approximately 14-fold less than the generation of NHDF-iPSCs (F-iPSCs, 0.7%). We obtained DPCs from four different patients. These DPCs all had significantly greater reprogramming efficiencies at 2.7% to 10.6%, a rate up to 15.1-fold increase compared to NHDFs. The quality of putative DPC-iPSC colonies (DP-iPSCs) generated was quantified by immunostaining for Nanog-positive colonies. On average, over 90% of the colonies generated from DPCs were Nanog-positive, compared to 64% of putative colonies from NHDFs. Interestingly, we were unsuccessful at generating iPSCs from genetically matched gingival tissue of one of our DPC samples. UCB-iPSCs, F-iPSCs, and DP-iPSCs all produced teratomas and had normal karyotype, phenotype and gene expression patterns. As inhibition of TGFβ activity can promote derivation of iPSCs, analysis of TGFβ activity may help prospectively assist in choosing optimal starting cell populations cells for reprogramming. We found expression of TGFβ receptors were significantly different between the starting cell populations, with DPCs having the lowest expression of TGFβ receptors. Next, we determined the ability of these different iPSCs to differentiate into hemato-endothelial cell populations. These studies using both stromal cell-mediated differentiation and embryoid body formation demonstrated the DP-iPSCs were less effective in differentiation into CD34+CD31+ hemato-endothelial cells, CD34+CD45+ hematopoietic cells or hematopoietic colony forming cells (CFCs), a measure of hematopoietic progenitor cells. Adding Wnt3a could improve hemato-endothelial cell development from DP-iPSCs. Taken together, although DPCs may be a robust source of adult cells to reprogram, DP-iPSC may have a reduced differentiation potential, making them less applicable for cellular therapies.

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MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF EARLY LINEAGE COMMITMENT OF HUMAN HEMATOPOIETIC STEM CELLS

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The hematopoietic system is a highly regulated cellular hierarchy, responsible for the day-to-day production of mature blood cells which can be divided in two major lineages, myeloid and lymphoid. Hematopoietic stem cells (HSCs) have the unique ability to give rise to all hematopoietic cell types, by first generating lineage-committed progenitors which in turn will produce terminally differentiated cells. HSCs are characterized by their extensive self-renewal and differentiation capacities. While in mice the mechanisms underlying early HSC differentiation and lineage determination are well understood at the molecular level, very few transcription factors regulating lineage decisions have been identified in human hematopoiesis. Our group has recently established a novel cell sorting strategy for human HSCs and early lineage committed progenitors (Doulatov et al., Nature Immunology; Notta et al., submitted) which uncovered the existence of a novel human multilymphoid progenitor (MLP). MLPs give rise to all lymphoid cell types, as well as dendritic cells and monocytic cells. Here we report a comprehensive analysis of gene expression at each developmental stage of the human hematopoietic hierarchy. We show that hematopoietic specification is defined by a small number of global gene expression clusters that correspond to major biological lineages and that lineage programs in committed progenitors are paired with HSC-shared priming programs. HSCs display most extensive priming along the lympho-myeloid branch (MLP). In contrast early progenitors of the megakaryocytic/erythrocytic lineage form a distinct cluster, highly enriched for cell cycle genes. Upon assembly of the transcription factor networks underlying each major developmental expression pattern, we selected a few candidate genes predicted by our model to be key regulators of lineage choices. As a proof of principle, we investigated the function of BCL11a, which expression is primed in HSCs then peaks in the newly discovered MLP stage. BCL11a is a C2H2 zinc finger transcriptional repressor, which has been implicated in the development of B cell progenitors in mouse. When BCL11a is knocked down in cord blood derived hematopoietic stem cells and early progenitors, we observe reduced formation of cells committed to the B cell fate both *in vitro* and in an *in vivo* xenograft assay. BCL11a knock-down resulted in a partial block of B cell maturation at the proB to preB cell transition that was accompanied by a decrease in the key B cell maturation transcription factor, Pax5. These preliminary results suggest that BCL11a directs B cell specification in human and that our genome-wide strategy allows identification of key regulators of human hematopoietic stem cells and of human lineage commitment.

Poster Board Number: 2340

RGS13 NEGATIVELY REGULATES SDF-1-DIRECTED MOTILITY IN HUMAN CORD BLOOD CD34+ HEMATOPOIETIC STEM/PROGENITOR CELLS

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The stromal cell-derived factor-1 (SDF-1)/chemokine C-X-C receptor 4 (CXCR4) axis is an essential mediator of hematopoietic stem cell (HSC) homing. In a recent study, we reported the transcriptome profile of human cord blood CD34+ cells in response to a short-term exposure of SDF-1 (4 hours; Leung KT et al., Blood, 2010). From the list of differentially regulated genes, we found that the expression level of 3 of the R4 Regulator of G-protein signaling (RGS) family proteins (RGS1, RGS2 and RGS13), were upregulated upon SDF-1 treatment (> 1.5-fold change; $P < .05$; $n = 4$). RGS family proteins are known to negatively control G-protein coupled receptor-evoked signal transduction through their GTPase-accelerating activity. In several types of hematopoietic cells, the SDF-1/CXCR4 axis is subjected to regulation by members of the R4 subfamily (including RGS1, RGS13 and RGS16). In this study, we investigated the expression level and possible role of RGS13 in SDF-1 induced homing-related functions of primary cord blood CD34+ HSC. Quantitative RT-PCR analysis revealed that enriched cord blood-derived CD34+ cells expressed relatively high mRNA level of RGS1, RGS2 and RGS3, and moderate level of RGS5, RGS13, RGS16 and RGS18 ($n = 3$). Expression of RGS8 was barely detectable, while RGS4 and RGS21 expression was not detectable. A short-term exposure (1-24 hours) of CD34+ cells to SDF-1 (100 ng/mL) slightly increased the expression of RGS1, RGS2 and RGS3 (≤ 1.5-fold; $P < .05$; $n = 4$). In agreement with the microarray data, a 4-hour SDF-1 treatment significantly upregulated RGS13 expression (1.8 ± .06-fold; $P = .04$; $n = 4$). Preincubation of CD34+ cells with the CXCR4 antagonist AMD3100 resulted in an 83.3% inhibition of SDF-1-induced RGS13 expression, indicating that it is a CXCR4-dependent event. To investigate the role of RGS13 in homing-related functions, we introduced RGS13 siRNA into CD34+ cells by nucleofection. With over 50% reduction in RGS13 mRNA level, migration of RGS13 siRNA-transfected cells towards a SDF-1 gradient was significantly enhanced (28.2% increase, $P = .02$, $n = 5$), when compared with cells transfected with control, non-targeting siRNA. Interestingly, the cell surface level of CXCR4 on RGS13 siRNA-transfected cells, either in the presence or absence of SDF-1, was not significantly different from that in control siRNA-transfected cells, suggesting that the enhanced migratory efficiency in RGS13 siRNA-transfected cells may be CXCR4-independent. Other homing-related functions, including SDF-1-induced actin polymerization and calcium mobilization, were not significantly altered in cells with reduced RGS13 expression. Taken together, we provided the first evidence that RGS13 expression is subjected to regulation by the SDF-1/CXCR4 axis in cord blood CD34+ HSC, and RGS13 is functionally involved in modulating SDF-1-mediated migration. Further investigation of RGS13-transfected cells in the NOD/SCID mouse transplantation model is in progress.

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HUMAN HEMATOPOIETIC STEM CELLS IN COCULTURE WITH BONE MARROW STROMAL CELLS: MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE HEMATOPOIETIC STEM CELL NICHE.

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Introduction: Hematopoietic stem cells (HSCs) are located in the bone marrow (BM) in a specific microenvironment referred as the hematopoietic stem cell niche, which plays a pivotal role in regulating their survival, self-renewal and differentiation. In this specific microenvironment, HSCs interact with a variety of stromal cells including fibroblasts, endothelial cells, reticular cells, osteoblasts and adipocytes. Though several components of the stem cell niche have been identified, the regulatory mechanisms through which such components regulate the stem cell fate are still unknown. In order to address this issue, we investigated how mesenchymal stem cells, osteoblasts and adipocytes can affect the molecular and functional phenotype of HSCs and vice versa in a coculture system. Methods: We set up 3 different coculture systems: CD34+ cells purified from the Cord Blood (CB) in culture with human osteoblasts purified from the trabecular bone or with human CD146+ bone marrow stromal cells or with adipocytes differentiated from CD146+ bone marrow stromal cells. After coculture, CD34+ cells and the hematopoietic cell fraction were separated from stromal cells and analyzed by gene expression profiling and clonogenic assay to assess how coculture could affect the self-renewal and differentiation capacity of HSCs. Results: Our preliminary results showed that coculture of CB CD34+ cells with CD146+ mesenchymal stem cells induces a strong increase in the clonogenic capacity of CD34+ cells and a five-fold expansion of the CD34+CD38- progenitor pool. Moreover, in the long-term culture, CD146+ stromal cells seem to favour the differentiation of hematopoietic cells towards the granulocytic lineage at the expense of the monocytic one. Osteoblasts were able to induce an increase in the clonogenic capacity and an expansion of primitive progenitors cells comparable to those induced by mesenchymal stem cells, but, on the other hand, clonogenic assay results showed an increase of the macrophage colonies and a decrease of the erythorid ones. Gene expression profiling analysis allowed us to study which signaling pathways were activated in the hematopoietic cell fraction and in the stromal cell compartment after coculture. Such analysis enabled us to identify several cytokine-receptor networks and transcription factors that could be activated by coculture with stromal cells and could be responsible for the biological effects reported above. Conclusion: Our preliminary results show that human CD146+ mesenchymal stem cells and osteoblast are both able to increase the clonogenic capacity of CD34+ cells and are capable of maintaining the more primitive hematopoietic progenitors up to 2 weeks in culture. Moreover, human CD146+ stromal cells seem to favour the differentiation of hematopoietic cells towards the granulocytic lineage at the expense of the monocytic one, whereas osteoblasts are able to affect the differentiation capacity of CD34+ cells by favouring macrophage commitment at the expense of the erythroid lineage. Gene expression profiling allowed us to identify few candidate transcriptions factors and signaling pathways underlying the effect exerted by stromal cells on HSCs. An understanding of how the niche participates in the maintenance of hematopoiesis offers new opportunities for the development of novel HSCs ex-vivo expansion protocols that allow stem cells expansion without loss of 'stemness'.

Poster Board Number: 2344

SOURCING HUMAN BLOOD-DERIVED RAW MATERIAL: OPTIMIZATION, QUALIFICATIONS AND CONTROL

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Human cells and tissue are critical raw material for cell therapy and tissue-engineered products, as well as *ex vivo* gene therapy products. Quality of this living cellular raw material is a major determinant of final product characteristics, but is complicated by the inherent heterogeneity and inter-individual variability of living biologics. Inconsistent collection procedures amplify this biological variability, adversely affecting the manufacturing process from the outset. Controlling and qualifying the cell collection step is essential to minimizing operational sources of variability, and greatly increases the likelihood of success in manufacturing. HemaCare's core competency is apheresis collection, and, building on 33 years of experience, has addressed this need for optimized cell collection with a program for controlling and qualifying its apheresis procedures and collection sites, to supply human-derived blood components for development and qualification of novel cell and gene therapies, assays, and medical devices. HemaCare's apheresis program includes comprehensive staff qualification and training, documentation that supports its cGMP environment and programs to monitor effectiveness of equipment and procedures in accordance with an established quality system. Donor recruitment, screening and IRB-approved consents follow the requirements of Good Tissue Practices (GTPs). From 2006-2010, inclusive, a total of 55,262 apheresis procedures were performed, including collection of patient and normal-donor peripheral blood mononuclear cells, mobilized peripheral blood progenitor cells, plateletpheresis products, and therapeutic apheresis. In 2010 alone, 11,646 apheresis products were collected, many of which supported clinical studies spanning Phase I-Phase III, preclinical research, and, for the first time, commercial cell therapy applications. This reflects the evolution of cellular therapies.

Poster Board Number: 2346

IDENTIFICATION OF HUMAN STEM CELL REGULATORS THROUGH HIGH-THROUGHPUT FUNCTIONAL SCREENS

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As many tissues in the body, the blood system is organized as a hierarchy sustained by stem cells at the apex. Hematopoietic stem cells (HSCs) give rise to proliferative progenitors which in turn are responsible for the output of approximately 1E12 differentiated cells per day. The feature that distinguishes HSCs from their downstream progenitors is self-renewal, an essential asset to maintain the blood system throughout the lifetime of an individual. Our lab has shown that leukemia is also organized as a hierarchy, as primary acute myeloid leukemia cells, which are capable of generating leukemia in xenografts, can be purified and distinguished from those leukemia cells that lack this ability. However, little is known about the regulation of self-renewal in either HSCs or leukemia stem cells (LSCs). We developed a high-throughput approach to functionally asses candidate stem cell regulatory genes. Genes were selected from expression arrays comparing primitive versus more differentiated cord blood and leukemia cell populations. To study these

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candidate genes, 96 overexpression lentiviruses were created and mixed in pools of 8-12 lentiviruses. These pools were used to transduce human cord blood cells, which were then injected into immunocompromised mice to determine if self-renewal ability was altered. After 16 weeks of *in vivo* competition, the abundance of every gene was assessed by quantitative PCR and compared to the cells before injection. Several genes were significantly enriched including CD200, which is involved in immune evasion and has been reported to be a cancer stem cell marker, and MCF2L, which is known to become tumorigenic when activated. Currently we are carrying out a similar experimental strategy to assess whether the candidate genes play a role in a leukemic background. We will validate and analyze the function of 10-20 genes in detail, to yield a significant advance in our understanding of the mechanisms that regulate the key stem cell property of self-renewal. We aim to get a more comprehensive understanding of self-renewal regulation in normal and leukemia stem cells, and to reveal genes and pathways that are perturbed in the initiation and progression of neoplastic growth. This is an important step towards the goal of finding new targets that could be therapeutically exploited to eradicate leukemia.

Poster Board Number: 2350

ANTI-TUMOR ACTIVITY FROM ANTIGEN SPECIFIC CD8 T CELLS GENERATED *IN VIVO* FROM GENETICALLY ENGINEERED HUMAN HEMATOPOIETIC STEM CELLS

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The goal of cancer immunotherapy is the generation of a large, efficient, stable and self-renewing anti-tumor T cell population. Herein we present the generation of functional tumor specific human T cells *in vivo* from genetically modified human hematopoietic stem cells (HSC) using a mouse/human chimera model. HSC transduced with a vector expressing an HLA-A*0201 restricted melanoma specific T cell receptor (TcR) were introduced into BLT humanized mice. This resulted in the generation of a sizeable melanoma specific CTL population that without any additional manipulation limited and cleared human melanoma tumors both *in vivo* and *ex vivo*. Bone marrow reconstitution lasted throughout the duration of the experiment, suggesting a long-term repopulation capacity of transgenic HSC. This novel approach represents an important tool to better understand and optimize human immune response to melanoma, and potentially other types of cancer.

Poster Board Number: 2352

HUMAN ADENYLATE KINASE 2 DEFICIENCY INHIBITS HEMATOPOIETIC CELL PROLIFERATION AND DIFFERENTIATION ALONG THE NEUTROPHIL AND T-CELL LINEAGES

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Reticular dysgenesis (RD), an autosomal recessive form of human Severe Combined Immunodeficiency is characterized by the absence of blood neutrophils and T lymphocytes. This pathology is due to biallelic mutations in the adenylate kinase 2 (AK2) gene, resulting in the loss of AK2 protein expression. AK2 is a mitochondrial protein which regulates the homeostasis of cellular adenine nucleotides by converting ADP into ATP and AMP. In order to understand the precise role of AK2 in hematopoiesis, we have developed a RNA interference strategy through lentiviral-mediated gene transfer of AK2 short hairpin RNAs (shAK2). The knock-down of AK2 in human or murine hematopoietic stem cells (HSC) inhibits their capacity to form granulocyte colonies in methylcellulose and prevents them to generate mature polynucleated cells in liquid culture in the presence of G-CSF. We also determine the ability of shAK2-transduced HSC to differentiate along the T lymphoid lineage after co-culture on a OP9Delta1 stroma cell line. Our data demonstrated that the apparition of CD4+CD8+ cells was profoundly reduced in the presence of shAK2. To delineate the mechanism involved in this defect, we also studied the neutrophil differentiation of the HL60 promyelocytic cell line, following retinoic acid treatment. In this system, the absence of AK2 expression led to an arrest of neutrophil differentiation process, increased cell apoptosis and disrupt the mitochondrial membrane potential whereas AK2 defect does not inhibit monocyte differentiation. All these data suggest a novel mechanism regulating hematopoietic cell proliferation and differentiation.

Poster Board Number: 2354

AUGMENTING THE MAINTENANCE OF HUMAN HEMATOPOIETIC STEM CELLS IN MICE

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Comprehensive understanding of human hematopoietic stem cell (HSC) biology and hematopoiesis requires *in vivo* analysis. The generation of 'humanized mice' that support the maintenance and continuous blood cell formation of human origin, holds the promise to be a valuable tool for that purpose. Mature human hematopoietic cell types are frequently found in the bone marrow and spleen of immunodeficient mouse strains, including BALB/c Rag2-/- Il2rgy/- mice after the transplantation of human HSCs. However, the ability to achieve stable human HSC chimerism, which is required for sustained hematopoiesis over a long period of time, is still limited in this mouse strain. To facilitate stable human HSC engraftment, we generated a novel recipient mouse strain with functionally defective endogenous HSCs in combination with immunodeficiency. In this mouse strain transplanted human HSCs are retained in the bone marrow for thirty weeks, and mainly human but not mouse hematopoietic progenitor cells are present in the bone marrow, suggesting a high participation of human donor cells to the hematopoiesis in the recipient mice. Transplanted human HSCs efficiently generate cells of the B lymphoid, erythroid and myeloid

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lineages over a long period of time in primary and secondary recipient mice confirming the regeneration of human HSCs. Our novel recipient mice have a greatly enhanced ability to support human HSC maintenance *in vivo*, and may provide a very useful tool to increase our knowledge about human HSC biology.

Poster Board Number: 2356

CLONAL ANALYSES REVEAL THAT THE AGED MOUSE HEMATOPOIETIC STEM CELL COMPARTMENT ACCUMULATES MYELOID-BIASED STEM CELLS WITH MULTIPLE FUNCTIONAL DEFECTS

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Aging in the C57Bl/6 mouse is characterized by an increase in the size of the hematopoietic stem cell (HSC) compartment, a corresponding decrease in functional HSC frequency in part due to a homing defect, and a myeloid lineage skewing of differentiated cell output. These functional changes in the aged HSC pool have been commonly attributed to cellular aging, whereby HSCs gradually lose function due to accumulated cellular damage. However, the identification of separate populations of intrinsically lineage biased HSCs has raised a new hypothesis, whereby the clonal composition of the HSC pool, rather than individual HSCs, change with age. While data exists to support both hypotheses, most HSC aging studies do not evaluate the functional properties of individual HSCs, but rather compare populations of old and young HSCs, which precludes any investigation of heterogeneity within the HSC compartment. In this study, we set out to obtain a more complete and high-resolution understanding of age-related differences in the HSC compartment. We compared multiple functional parameters of individual HSCs from young and old mice, including their short-term marrow homing ability, *in vitro* proliferative activity, *in vivo* differentiation properties and *in vivo* self-renewal behaviour. We confirm an increase in the absolute number and relative frequency of myeloid-biased HSCs in old mice. However, the myeloid-biased HSCs from old mice were clearly different from those in young mice in that most had a very small output of differentiated blood cells when transplanted *in vivo*. Nevertheless, these limited-output myeloid-biased HSCs from old mice still had extensive self-renewal ability as measured by long-term secondary transplantation, albeit at a reduced rate compared to young HSCs. Additional functional differences that were observed by comparing purified old and young HSCs included a decrease in short-term *in vivo* homing efficiency and a reduction in clonogenic efficiency in stromal co-cultures. By testing these two properties sequentially on the same cells, we could conclude that these functional defects are not indicative of a non-functional subset of cells that express HSC markers, rather, they appear to be characteristics of all old HSCs. Old HSCs also exhibited a marked delay in proliferative response in stromal co-cultures and a reduced blood cell output in secondary recipients, regardless of their initial lineage bias. Furthermore, we show that *in vivo* repopulating cells with characteristics of aged HSCs can be generated from young HSCs over the course of multiple serial transplants with a timeframe similar to the lifespan of a mouse. In conclusion, the clonal composition of the aged HSC pool changes to become dominated by functionally defective HSCs that appear to arise from normal HSCs by a process of cellular aging.

Poster Board Number: 2358

LOSS OF ARYL HYDROCARBON RECEPTOR PROMOTES HEMATOPOIETIC STEM CELL AGING AND SENESCENCE, AND SIGNS OF LEUKEMIA IN MICE.

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The aryl hydrocarbon receptor (Ahr) is a ligand-activated basic helix-loop-helix gene regulatory protein that belongs to the Per-Arnt-Sim (PAS) superfamily. However, despite extensive studies, the normal physiological function of the Ahr is still not known. Our earlier studies indicated that juvenile Ahr-null allele (KO) mice have increased numbers of hematopoietic stem cell (HSC) enriched Lin-Sca-1+c-kit+ (LSK) cells in bone marrow, and these cells have high rates of proliferation and cell division as determined by increased BrdU incorporation and cell cycle analysis. Progenitor cells from these mice also have increased growth rates in culture (Singh et al., Stem Cells Dev, 2011). These data are consistent with a recent report indicating AHR antagonists promote the expansion of human HSCs. These and other reports suggest that the Ahr is a negative regulator of HSC proliferation by promoting HSCs to remain in quiescence. Based on these findings, we further hypothesized HSCs in aging Ahr-KO mice may undergo premature senescence leading to the development of hematologic disease. We examined phenotypic and functional changes in HSCs, progenitors, and lineage cells in two-year old KO and WT mice. KO mice showed increases in total bone marrow cells. In peripheral blood, there were increased total white blood cells and lymphocytes, but decreased red blood cells number. Analysis of bone marrow demonstrated increases in stem and progenitor cell populations, as well as increases in the colony-forming assays HPP-CFC and CFU-preB, but decreased BFU-E, CFU-E, CFU-M, and CFU-S8. Functional analysis of stem cells indicated a significant decrease in competitive repopulation units following limiting-dilution analysis. Furthermore, HSCs from KO mice demonstrated lower competitive repopulation ability following primary, secondary, and tertiary serial transplants suggesting compromised self-renewal and differentiation. Tissues from aging KO mice demonstrated abnormal pathological changes with substantially increased lymphocytic infiltration in spleen, liver, and lung. Use of pathway-specific RT-PCR arrays showed alterations in spleen cell gene expression for a number of chemokines, cytokines and their receptors, as well as inflammatory related genes. The expression of several leukemia associated genes, including gata-1, S2d3C, and gfi-1 were down regulated in spleen cells. Ahr-KO mice also demonstrated significantly lower survival rates, with an approximate 6-month difference in lifespan. These data, in combination with our previous report, indicates that a lifetime of increased cycling by HSCs lacking a functional Ahr will eventually lead to premature HSC aging and senescence. Furthermore, loss of Ahr expression and altered HSC function predisposes these mice to the development of a significant hematopoietic disorder that is consistent with signs of leukemia. These data further support our contention that the Ahr is a negative regulator of HSC proliferation - a novel physiological role for this transcription factor. Ultimately, it appears that the Ahr regulates critical genes and signaling pathways that allow HSCs to differentially respond to signals in their microenvironment. However, additional work is clearly needed to define these relationships and the signaling pathways responsible for them. (Funded by NIH Grants ES04862, ES016606, Center Grant ES01247, and Training Grant ES07026.)

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CCN3(NOV) CONTRIBUTES TO THOROMBOPOIETIN-MEDIATED MAINTENANCE OF MOUSE HEMATOPOIETIC STEM CELLS

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Through life, the supply of all blood cells depends on sustained division of hematopoietic stem cells (HSCs) for self-renewal and multi-differentiation potency. HSCs usually reside in a specialized microenvironment termed "niche", where HSC functions are regulated. Although it is generally proposed that cells around HSCs, such as osteoblasts or vascular (sinusoid) endothelial cells form their niches, the contribution to forming their own niche by HSCs still remains unclear. Herein, our study showed that HSCs-derived soluble factors (HSFs) regulated proliferation of HSCs. Firstly, we examined whether HSFs affect HSC functions, since our gene expression assay using whole transcriptome showed that CD34- c-kit+ Sca-1+ Linage marker- (CD34-KSL) HSCs enhanced expression of several soluble factors which was reportedly involved with HSC regulation, compared to CD34+KSL hematopoietic progenitors. Therefore, to examine the effect of HSFs on HSCs, we utilized the culture system using a cell culture insert in the presence of stem cell factor (SCF) and thrombopoietin (TPO). Actually, bone marrow transplantation (BMT) assay following the culture showed the positive effect of HSFs on HSC proliferation. Importantly, the conditioned medium obtained from cultured HSCs with stem cell factor (SCF) and thrombopoietin (TPO) enhanced HSC proliferation, but not that from uncultured fresh HSCs. These results suggest that SCF and/or TPO stimulation on HSCs is required for the production of HSFs which demonstrate the positive action for HSC proliferation. Therefore, we next examined soluble factors in which mRNA expression was enhanced on HSCs by the culture with SCF and TPO on HSCs using SOLiD sequencing, resulting in the extraction of 6 genes containing Nov/CCN3, which was reportedly essential for the maintenance of human HSCs. Actually, we also confirmed the positive effect of Nov on mouse HSC activity by BMT assays. Because enhanced expression of Nov on HSCs is observed in the presence of SCF and TPO, we tested whether SCF and/or TPO preferentially contribute to expression of Nov by quantitative Real-time RT-PCR following the culture in the presence SCF and/or TPO. Importantly, TPO enable to induce enhanced expression of Nov on HSCs, while SCF completely failed. Therefore, these results show that expression of Nov by HSCs is dependent on the presence of TPO, suggesting that Nov contributes to TPO-dependent maintenance of HSC activity. Actually, BMT assays demonstrated the positive action of Nov on HSC activity only in the presence of TPO, supporting this possibility. In addition, we found that TPO, but not SCF, progressed Nov binding to HSCs via undetected receptors in flow cytometric analysis using Alexa fluor 647-labed Nov. Thus, this may elucidate TPO-dependent effects of Nov on HSC activity. In summary, our findings show a novel mechanism of the TPO-dependent maintenance of HSC activity via Nov. Moreover, we also demonstrate that HSFs including Nov are involved with HSC regulation, which suggests the possibility that HSCs also contribute to forming their own niche through the production of effective soluble factors for HSC regulation.

Poster Board Number: 2362

IMMUNE REGENERATION MEDIATED BY ENHANCED MOUSE HEMATOPOIETIC STEM AND PROGENITOR CELL FUNCTION

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Discordant with its importance, the thymus undergoes severe involution with age, characterized by profound architectural disorganization and significant decline in thymocyte production and output. As optimal T cell development requires a functional thymus, patients who suffer from immunodepleting conditions (e.g. cytoreductive therapies, HSCT, or AIDS) have poor immune recovery, in part due to their declining thymic function. We have previously found that ablation of sex steroids (SSA) restores thymic function and bone marrow (BM) lymphopoiesis. In the present study, we examined the impact of SSA on BM and thymic hematopoietic stem and progenitor cells (HSPC). SSA significantly enhanced the most primitive HSC population both quantitatively as well as functionally. This was demonstrated by their lymphoid-lineage potential *in vitro* and *in vivo*, as well as their self-renewal capacity post SSA. These findings suggest a potential involvement in the fundamental mechanism underlying SSA-mediated immune regeneration. In addition we also found that molecular changes in the BM niche make it more conducive for lymphoid commitment and lymphopoiesis. While there is considerable debate surrounding the identity of the earliest BM-derived T cell progenitor, it is becoming increasingly clear that several phenotypically distinct progenitors have the ability to seed the thymus and initiate thymopoiesis. We found that following SSA there is a quantitative increase across the full spectrum of BM-resident and intrathymic T-lineage progenitors with their T-lineage differentiation potential also significantly enhanced. Moreover, in a series of short-term *in vivo* homing assays, we found that while SSA significantly increased intrathymic niche availability, non-specifically attracting both canonical and non-canonical T-lineage progenitors, SSA also selectively improved the ability of non-canonical T-lineage progenitors to enter the thymus. Together these findings provide a tantalizing glimpse into improving immune function - a critical step in improving clinical outcomes from chemotherapy, HSCT, or other immune depleting therapies.

Poster Board Number: 2364

TYPES I AND II INTERFERON COORDINATELY REGULATE MURINE HEMATOPOIETIC STEM CELLS DURING HOMEOSTASIS AND ACUTE VIRAL INFECTION

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The role of the hematopoietic stem cell (HSC) in the primary immune response to infection is unknown. We have recently demonstrated that HSCs proliferate and mobilize to the periphery in response to systemic chronic Mycobacterium avium infection in mice. Interferon-gamma (IFNγ) signaling through the transcriptional regulator Stat1 is both necessary and sufficient to mediate this response. Interferon-alpha (IFNα) can mediate similar cell-autonomous stimulatory effects on HSCs. We hypothesized that IFNα and IFNγ fulfill redundant roles in activating quiescent HSCs during infection. In order to assess the relative importance of IFNα and IFNγ, we utilized a mouse model of acute viral infection. Lymphochoriomeningitis virus (LCMV) infection generates a mixed IFNα and IFNγ response in host animals. We

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ascertained that HSCs are activated to proliferate in response to acute LCMV infection. Next, we assessed the response of HSCs to LCMV infection in mice lacking interferon-alpha receptor 1 (Ifnar1), interferon-gamma receptor 1 (Ifngr1), or Stat1, a transcription factor shared by both IFNα and IFNγ signaling pathways. In addition, we generated double knock out animals lacking both Ifnar1 and Ifngr1, which we predicted would show a phenotype similar to the Stat1-deficient mice. HSC expansion in response to viral infection was achieved in LCMV-infected animals lacking either Ifnar1 or Ifngr1, but not both. The response of HSCs from Ifnar1-/- Ifngr1-/- double knock-out and Stat1-/- animals was similar, as predicted. We also performed transplantation experiments to test the relative reconstitution potential of bone marrow of the mutant mice. In the absence of infection, reconstitution potential was maintained or improved in the marrow of Ifnar1-deficient or Ifngr1-deficient animals, but diminished in bone marrow of Ifnar1-/- Ifngr1-/- double knock-out and Stat1-/- animals. These findings suggest that HSCs can be activated in response to both acute and chronic infections. Furthermore, Types I (IFNα) and II (IFNγ) interferons serve redundant functions in triggering HSC stimulation in the context of LCMV infection, and they participate in maintenance of HSC reconstitution potential at baseline. These findings contribute to our growing understanding of the role of cytokines in affecting HSC function. We postulate that direct stimulation of HSC proliferation may be important in HSC homeostasis as well as in tailoring the immune response during infection. Furthermore, interferon-based responses may contribute to syndromes of HSC malfunction in clinical contexts such as post-transplant engraftment, bone marrow failure syndromes, and acquired aplastic anemia following infection. Further study of immune regulation of HSCs will provide insight into these clinical syndromes as well as the long-term effects of inflammation on bone marrow function.

Poster Board Number: 2366

PODOCALYXIN ENHANCES CXCL12-MEDIATED MIGRATION OF MOUSE HEMATOPOIETIC CELLS

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CD34, podocalyxin and endoglycan comprise a family of cell surface sialomucins with overlapping pattern of expression on hematopoietic precursors and vascular cells. Although CD34 has long been used in the clinic as a marker of hematopoietic progenitors with long-term repopulation potential, little is known of the functions of CD34 sialomucins *in vivo*. In attempts to define CD34-family functions, CD34-sialomucins have been shown to mediate pro- or anti-adhesive functions related to tissue architecture or cell trafficking in both hematopoietic and non-hematopoietic cell types. However, the molecular mechanisms underlying these putative functions remain enigmatic. We have found that CD34 and podocalyxin promote the homing of definitive, fetal hematopoietic cells to the bone marrow of recipient mice in short term homing assays. To better characterize the mechanisms underlying this function, we have used shRNA lentiviral vectors to stably knockdown podocalyxin (Podxl) expression in the IL-3 dependent mouse hematopoietic precursor cell line, FDC-P1. In IL-3-deprived wild type FDC-P1 cells, podocalyxin becomes membrane-polarized in response to treatment with IL-3 or stem cell factor (SCF) + CXCL12. In addition, although we only find low levels CXCR4, a CXCL12 receptor, on the surface of FDC-P1 cells at steady state, CXCR4 surface expression increases rapidly in response to SCF + CXCL12 treatment and CXCR4 co-polarizes with podocalyxin. Strikingly, Podxl-deficient FDC-P1 cells fail to upregulate surface expression of CXCR4 in response to SCF + CXCL12 and are severely impaired in their ability to migrate to SCF or CXCL12. Podxl-/- fetal liver progenitors, likewise, exhibit an impaired ability to migrate to SCF + CXCL12 *in vitro*. These data suggest that podocalyxin enhances chemokine-dependent migration, perhaps by stabilizing the appropriate polarized expression of chemokine receptors on the cell surface. These results offer the first mechanistic explanation for the cell-trafficking functions associated with the CD34-family sialomucins.

Poster Board Number: 2368

A NOVEL FUNCTION OF DEVELOPING MURINE ENDOCARDIUM

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We have previously demonstrated that single cardiac progenitors expressing Flk1 Isl1, and Nkx2.5 give rise to both cardiac and endocardial/endothelial cell lineages during early murine cardiogenesis. Interestingly, the pool of Flk1+/Isl1+/Nkx2.5+ cardiac progenitors express multiple hematopoietic transcription factors. Although a close relationship between cardiac, endocardial, and hematopoietic lineages has been suggested in *Drosophila*, zebrafish, and embryonic stem cell *in vitro* differentiation models, the significance of hematopoietic genes in the developing mammalian heart remains unclear. Here, we demonstrate hemogenic activity in the developing murine endocardium. A subset of developing endocardium expresses a hematopoietic progenitor marker, CD41, proliferates *in situ*, and emerges into the cardiac chamber. FACS-purified endocardial cells are capable of generating blood colonies *ex vivo*. In search for the molecular mechanism underlying hematopoiesis in endocardium, we identified Nkx2.5 as a key regulator. Nkx2.5 mutant heart showed almost no CD41 positive cells in the endocardium, and Nkx2.5 null ES cells generated significantly less blood cells than the control ES cells. These data, taken together, suggest that developing murine endocardial cells display hemogenic activity during cardiogenesis via an Nkx2.5-dependent pathway.

Poster Board Number: 2370

SLAM FAMILY MARKERS IMPROVE THE ABILITY TO DISTINGUISH DIFFERENT SUBPOPULATIONS OF MOUSE MULTIPOTENT HEMATOPOIETIC PROGENITORS

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Various combinations of markers have been used to isolate transiently reconstituting multipotent hematopoietic progenitors (MPPs); however, all such populations remain heterogeneous, containing different subpopulations of MPPs with different degrees of reconstituting capacity as well as restricted progenitors. Hematopoietic stem cell (HSC) and MPP purity has been enhanced using two SLAM family markers: CD150 and CD48. The Lineage-Sca-1+c-Kit+ (LSK) hematopoietic stem and progenitor fraction can be separated into a CD150+CD48-LSK HSC fraction, a CD150-CD48-LSK MPP fraction, and two downstream hematopoietic progenitor cell (HPC) fractions, CD150-CD48+LSK HPC-1 and CD150+CD48+LSK HPC-2. To elucidate a detailed functional hierarchy of HSC and MPP fractions, we identified additional SLAM family markers that can further subdivide primitive hematopoietic progenitors: CD229 and CD244. These new markers can break down HSCs into two sub fractions (CD229-CD244- HSC-1 and CD229+CD244- HSC-2) and MPPs into three sub fractions (CD229-CD244- MPP-1, CD229+CD244- MPP-2, and CD229+CD244+ MPP-3). These two HSC fractions, three MPP fractions, and two HPC fractions possess

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differential cell-cycle status revealed from BrdU incorporation, Ki67 staining, and histone H2B-GFP retaining assays. Among these fractions, HSC-1 is the most quiescent fraction, with HSC-2 dividing more actively than HSC-1. The progenitor fractions showed increasing cell cycle activity in this order: MPP-1, MPP-2, MPP-3, HPC-1, and HPC-2. Bone marrow (BM) reconstitution assays with very small numbers of cells showed that both HSC fractions have long-term multi-lineage repopulating capacity. However, serial transplantation assays revealed that HSC-1 robustly repopulated secondary recipient BM, whereas HSC-2 poorly repopulated secondary recipients. Most MPPs gave transient multilineage reconstitution and the duration of reconstitution declined in this order: MPP-1, MPP-2, and MPP-3 (longest to shortest). These findings demonstrate the existence of a functional hierarchy within HSC and MPP fractions, and the ability to distinguish functionally distinct subpopulations of HSCs and MPPs using SLAM family markers.

Poster Board Number: 2372

CORRECTION OF ARTEMIS DEFICIENCY IN MURINE HEMATOPOIETIC STEM CELL BY I-SCE1 MEGANUCLEASE AND ARTEMIS RECOMBINATION MATRIX MEDIATED HOMOLOGOUS RECOMBINATION.

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Gene therapy allows correction of a number of monogenic diseases affecting the hematopoietic system. This approach can be conducted by two different strategies which are either semi-random integration of the therapeutical gene using self-inactivating (SIN) retroviral vectors or homologous recombination (HR). The feasibility and efficacy of the first approach has been demonstrated in different forms of severe combined immunodeficiency (SCID) (SCID-X1 and Adenosine Deaminase) but the occurrence of a number of severe adverse events leads to further consider the HR approach. Homologous recombination (HR) is a highly specific DNA-repair mechanism, allowing to selectively replace a DNA sequence by another of interest. DNA double strand breaks (DSB) can enhance the frequency of HR. Specific DSB can be induced by meganucleases. These meganucleases are endonucleases with long DNA-recognition sites (12-30bp). They thus principally allow to target gene specific positions in the genome. In view of that, engineering gene specific meganucleases represents a tempting novel approach for gene therapy and targeting. To further develop this approach, we chose the Artemis deficiency model. This variant of SCID combines the phenotype of radio-sensitivity and lymphocyte developmental arrest at the double negative CD44-CD25+ T-progenitor and the B220+CD43+ B-progenitor stage. In this model, successful gene targeting can thus be assessed by reversion of the pathological phenotype in a number of assays used to study radiosensitivity, B- or T-cell development *in vitro*. The Artemis deficient mouse model was created by substitution of exon 12 of the murine Artemis gene by a restriction site for the I-Sce 1. The I-Sce 1 specific meganuclease would thus allow site specific induction of DSB at the Artemis gene. To deliver the I-Sce 1 meganuclease and to provide the Artemis DNA matrix we designed two SIN-integrase-defective lentiviral vectors (SIN-IDLV): IDLV-CMVI-Sce 1 and IDLV-Art. SIN-IDLV were used to avoid non-specific integration and transgene over-expression. Transduction of bone marrow derived Sca1+ hematopoietic stem cells (HSC) from Art-/- mice with both vectors, IDLV-CMVI-Sce 1 and IDLV-Art, resulted in a transduction efficiency of about 30%. Reversion of the Artemis deficient phenotype indicating successful gene targeting of the Artemis exon 12 was observed in 2 out 10 experiments by means of appearance of CD4/CD8 double positive T cells after co-culture on the OP9-DL1 system. Site specific insertion of Artemis exon 12 by HR was confirmed by nested-PCR and genomic DNA sequence analysis. These preliminary results demonstrate that meganucleases and repair matrixes can induce site specific DNA-repair by HR in HSC. Even though the efficiency of genomic reparation needs to be further improved and its potential genotoxicity remains to be evaluated, this new approach of gene therapies is very promising for the future.

Poster Board Number: 2374

THE SKP2 E3 LIGASE IS REQUIRED FOR MOUSE HSC REGENERATION IN RESPONSE TO MYELOABLATIVE STRESS AND BONE MARROW TRANSPLANTATION.

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SKP2 is the F-box subunit of the ubiquitin-ligase complex SCFSKP2 that targets the cyclin-dependent kinases (CDKs) p27Kip1, p21Cip1, p57Kip2 and p130 for ubiquitination and degradation. SKP2 protein levels are modulated during cell cycle and in response to mitogenic stimuli: its expression is essential for cell cycle entry whereas its downregulation is critical for cell cycle arrest. The fact that SKP2 controls the destruction of several CKIs underscores its central role as a cell cycle regulator. Alteration of the CKIs p21Cip1 and p27Kip1 levels has a significant impact on hematopoietic cells; however, the role of SKP2 in hematopoiesis has not been investigated, and it is not known whether SKP2 regulates these CKIs in distinct hematopoietic subpopulations. We found that SKP2 is present in murine bone marrow (BM) HSC and progenitors, and that its expression is dynamically regulated in response to hematopoietic stress requiring rapid cell cycle entry. To better define the role of SKP2 in hematopoietic homeostasis, we analyzed the effects of SKP2 deletion in the BM compartment of SKP2 null mice. At steady-state, SKP2 deletion decreased the mitotic activity of HSC and progenitors resulting in enhanced HSC quiescence and increased pool size. Immunophenotypical analysis of the distinct BM subsets showed that SKP2 deletion resulted in a significant increase in HSC (LT-HSC and ST-HSC) both in percentages and absolute numbers (LSK, averages: 6.6% KO vs 3% WT vs; $p<0.001$). Conversely, the pool of highly cycling erythromyeloid progenitors were significantly decreased in the absence of SKP2 (GMP; averages 2.4% KO vs 4.2% WT; $p=0.03$; MEP; averages 9% KO vs 14% WT; $p=0.01$). As anticipated, loss of SKP2 expression correlated with the accumulation of p27Kip1, p57Kip2 and p130 in SKP2 -/- HSC, and with overall reduced cell cycle, as determined by *in vivo* BrdU incorporation. Next, we determined whether the loss of SKP2 have a negative impact on HSC ability to enter the cell cycle in response to stress. Competitive repopulation assays demonstrated that SKP2-/- stem/progenitor cells were greatly impaired in reconstituting hematopoiesis at short-term (4th week CD45.2: 30% in KO vs 60% in WT vs; $p=0.001$). In agreement with these observations, SKP2-/- mice showed a marked slower hematopoietic regeneration following 5-FU myelosuppression, as indicated by lower WBC counts (50% less at week 2) compared to WT mice. As anticipated, long-term reconstitution (>20 weeks) was not affected or slightly improved in mice transplanted with SKP2-/- cells. Thus, SKP2 deficiency results in a slower cell cycle that: i) at steady-state favors quiescence in HSC, and results in decreased proliferative rates in progenitors; and ii) following stress impairs both HSC and progenitors to enter rapidly in the cell cycle. Taken together, these data indicate that SKP2 is a physiologic regulator of cell cycle progression and regeneration of HSC and progenitors and provide new insights on the mechanisms involved in BM recovery post-chemotherapy and radiation and BM transplantation.

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EPIGENETIC REGULATION OF IMPRINTING GENE INFLUENCES THE QUIESCENT VERSUS ACTIVE STATE AND FUNCTION OF ADULT MOUSE HEMATOPOIETIC STEM CELLS

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There is compelling evidence that hematopoietic stem cells (HSCs) exist in an active (enriched in short term HSC) and quiescent (enriched long term HSCs) state. Earlier studies from our laboratory revealed differential expression of imprinting genes in these two populations, These observations led us to hypothesize that the epigenetic state of imprinting genes can influence HSC state and its function. To test this hypothesis, we used a genetic approach complemented by functional analysis. Mice with a conditional deletion of the differential methylation region (DMD), 2kb upstream of the 5' prime region of the imprinting gene H19 were employed. Since imprinting genes are known to be mono-allelic in expression, female & male H19fx/fx were crossed with Mx cre mice of the opposite gender to generate mice with maternally & paternally deleted DMD alleles (H19ΔDMD) respectively. Unlike deletion from the paternal allele (H19ΔDMD), that showed no difference in frequency of LT HSC in BM, deletion from the maternal allele resulted in phenotypic reduction in LT HSCs (LSK CD34-Flk2-) accompanied by an increase in ST HSCs (LSK CD34+Flk2-) and multi progenitor(MPP) cells(LSK CD34+Flk2+). The competitive repopulation assay confirmed compromised function of HSC in the maternal inherited H19 Δ DMD mutant mice. This data suggests that the epigenetic state of imprinting genes regulates stem cell state and function. Deletion of the DMD region was accompanied by a decrease in H19 gene expression and reciprocal up regulation of Igf2, leading to exhaustion of LT HSC as well as the LSK population by 16 weeks post deletion. In conclusion, using a combination of genetic and functional approaches our data clearly demonstrate that epigenetic regulation of imprinting genes regulate HSC state and function. We anticipate that this mechanism of regulation may also exist in other types of adult somatic stem cells.

Poster Board Number: 2378

IN VIVO MARKING DEMONSTRATES LONG-TERM CONTRIBUTION OF MULTIPLE STEM CELL CLONES TO MURINE STEADY-STATE HEMATOPOIESIS

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Distinct hematopoietic stem and progenitor cells are defined by their role in post-transplant reconstitution as the analysis of the properties of isolated stem cells required syngeneic or xenogeneic transplantation models. Therefore, the clonal activity of individual hematopoietic stem and progenitor cells in undisturbed steady-state hematopoiesis is poorly understood. To address the clonal contribution of individual hematopoietic stem cells under steady-state conditions, murine hematopoietic stem and progenitor cells were directly marked by intrafemoral (IF, n=10) or intravenous (IV, n=5) injection of GFP-expressing lentiviral vectors (LV) into eGFP-tolerant B6.Cg-Tg (Krt1-15-EGFP) mice (Krt15). GFP expression in murine peripheral blood (PB) was monitored for up to 24 months after *in vivo* marking and myeloid and lymphoid blood cells were isolated by fluorescence activated cell sorting. A low but remarkably stable proportion of myeloid and lymphoid hematopoietic cells expressed GFP for up to 24 months (5-100 GFP+ cells per 20000 PB cells analyzed), indicating stable marking of murine steady-state hematopoiesis. Long-term contribution of marked cells in PB showed a comparable distribution into myeloid and lymphoid cell lineages. Pre-conditioning with 5-Fluorouracil did not influence the proportion or lineage distribution of marked hematopoiesis (IF: n=5; IV: n=5). Although IV-Injection of LV resulted in comparable percentages of marked cells as IF injection ($p>0.05$) the marking kinetics were different. While the proportion of marked cells remained stable for up to 2 years after IF Injection, IV-marking resulted in a 2-fold decline of GFP expressing cells during the first two weeks after IV-marking indicating that IV vector administration primarily targets short-lived more mature cells. Monitoring of individual marked clones in sorted cell fractions by highly sensitive amplification of lentiviral integration sites using LAM-PCR demonstrated long-term contribution of marked clones to myeloid and lymphoid hematopoiesis. 1-10 marked clones detected by LAM-PCR generated only 0.01%-0.23% of all PB cells in individual mice suggesting that at least 750 to several thousand hematopoietic clones simultaneously contribute to steady-state hematopoiesis. Immunostaining of histological sections 24 months after transplantation verified that eGFP expressing hematopoietic cells were located in the bone marrow. In addition, marked non-hematopoietic and hematopoietic cells were found in liver and spleen of injected mice. In summary, these data demonstrate stable long-term marking of undisturbed steady-state hematopoiesis for up to 24 months. We show that stem cell clones with myeloid and lymphoid differentiation potential stably contribute to murine steady-state hematopoiesis. These studies provide evidence that an unexpected high number of individual stem cell clones are active in murine steady-state hematopoiesis. *In vivo* marking further allows to directly analyze the influence of hematopoietic stress such as chemotherapy to individual stem cell clones.

Poster Board Number: 2380

STEM CELLS ENHANCE MOTOR RECOVERY FOLLOWING STROKE IN LONG-EVANS RATS

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Background: Stroke is the leading cause of disability in the Unites States resulting in upper extremity motor impairments. Methods: Male Long-Evans hooded rats (350-420g) underwent stroke surgery using Endothelin-1 induced MCAO. Animals were housed in standard laboratory cages on a 12 hour light/dark cycle. Animals were pretrained to familiarize them with the reaching accuracy task. Trained animals were then divided into 3 groups (controls, n=7; stroke, n=3; stroke with post-reperfusion administered endothelial progenitor cells (EPC's), n=6); The EPC's were enriched from the bone marrow of a separate group of donor rats using nanoparticles tagged with LIN negative and CD90 markers. Ten million EPC's were injected into the tail vein immediately following reperfusion. Animals were assessed at 3 and 5 weeks following the surgery for reaching task performance. All assessments were performed in a blinded manner. Results: Reaching accuracy: Control animals scored a reaching accuracy of 41±8.1% and 46±11% at 3 and 5 weeks, respectively. Stroke decreased this task to 12±21% and 18±28%, respectively ($p<0.05$, compared to controls at both time points). Injection of EPC's following reperfusion led to an increase in reaching accuracy to 27±26% and 32±24%, at 3 and 5 weeks, respectively ($p<0.05$, compared to stroked non-EPC cohort at 5 weeks). Conclusions: These data suggest that Hematopoietic EPC's play a significant role in the amelioration of stroke injury following cerebral ischemia.

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HOXB4 INDUCES MORE SELF-RENEWAL ACTIVITY IN CD133 POSITIVE RATHER THAN CD34 POSITIVE HEMATOPOIETIC STEM CELLS

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Background: because of several advantages of cord blood HSCs over other sources of HSCs, They increasingly attract attention for BM transplantation purposes. But unfortunately the number of HSCs is a limiting factor for BM transplantation in adult recipients. Cord blood HSCs *ex vivo* expansion of cord blood HSCs is investigated as an beneficial approach to overcome the problem by HSCs self-renewal induction. Meanwhile HOXB4 has been shown its powerful capability for HSCs self-renewal induction. In this study we evaluated and compared the response of CD34+ and CD133+ HSCs as different subpopulations of HSCs to HOXB4 self-renewal induction. Material and methods: HOXB4 was cloned in pLEX-MSC lentiviral vector. Then we produced lentiviruses containing HOXB4 and transduced cord blood CD34+ and CD133+ HSCs isolated with MACS column. HSCs were cultured in IMDM medium containing 10% FBS and early acting cytokines (Flt3L, SCF, Tpo) for 8 days. During *ex vivo* expansion period we evaluate HOXB4 over Expression using QRT-PCR and thereafter HOXB4 protein synthesis was evaluated by western blotting. By the end of 8th day, CD133+ and CD34+ subpopulation of cultured cells were examined by flow cytometric analysis. Finally the expanded cells were evaluated for the presence of early hematopoietic stem cells by LT-CIC and clonogenic assays. Results: our results showed that HOXB4 increase self-renewal of both CD34+ and CD133+ HSCs subpopulation as it was shown by flow cytometric analysis. Then we decided to assess the frequency of LT-CICs by LT-CIC test. We saw that CD133+ HSCs showed more LT-CIC frequency than CD34+ HSCs. Discussion: altogether our results showed that CD133+ HSCs transduced with HOXB4 lentivirus produced more long term initiating cells in LT-CIC test and it shows that CD133+ HSCs are possibly better targets for HOXB4 gene delivery for self-renewal induction and produces more valuable cell resources for BM transplantation.
Key words: HSCs, HOXB4, LT-CIC

Poster Board Number: 2384

DEVELOPMENT OF AN EXPANSION MODEL OF CD34+ UMBILICAL CORD BLOOD CELLS COCULTURE IN FEEDER LAYERS OF MESENCHYMAL CELLS AND CYTOKINES.

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Umbilical Cord Blood (UBC) has CD34+ Hematopoietic Stem cells (HSCs), which have the ability of long term bone marrow restocking. The blood is easily obtained and criopreserved for posterior use, which makes it a feasible alternative for transplant. The limited volume of obtained UCB yields low counts of HSCs, having a repercussion on the probability of doing a short-term graft. Around the world, scientists are trying to solve this by increasing the number of HSCs *ex vivo* with human growth factors, for its posterior grafting. It has been proven that for the homing of the HSCs the expression of CXCR4 receptor is required which interacts with Stromal Cells Derived Factor 1 (SDF-1/CXCL12) that stimulates signal pathways for cellular division, proliferation and chemotaxis. This research developed a small scale model for the expansion of HSCs using the human recombinant cytokines (HRC), Stem Cell Factor (SCF), Interleukin 3(IL-3), thrombopoyetin (TPO) and FT3 ligand (FLT3) (Cell Genix Germany®); altogether with Mesenchymal Stem Cell feeding cultures obtained from protein-free animal supplements, which where compared with previously established models. Those CD34+ cells cultured with the developed model, using supplement processed lysate platelet-rich human plasma, showed an initial expansion factor of 7.7±2.2 times ($p<0.05$) greater compared with their initial value. This value was higher compared with the other studied conditions, showing increasing numbers after day 12 of culture ($p<0.05$).The expression of CXCR4 was significantly higher in the model when compared with other studied models, the obtained value was 51.324±2.436 cells ($p<0.05$),and therefore it showed a tendency for an increase in this receptor expression over time. However, when comparing the CD34+ cell population against those CD34+/CXCR4+, we obtained an expression of the CXCR4 on nearly 70% of the cell population, considering all the conditions studied; suggesting that the proposed model using mesenchymal cells as support, permits the necessary interaction with CD34+ cells needed not only for division but also for the preservation of the homing and chemotaxis molecules. Therefore, this study shows evidence for an efficient model of CD34+ cells expansion.

Poster Board Number: 2386

ENDOGLIN IS NECESSARY FOR PROPER HEMATOPOIETIC DEVELOPMENT

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Endoglin (Eng), an accessory receptor for the Transforming Growth Factor (TGF)-beta superfamily, plays a critical role in early development. Endoglin-deficient mice die around 10.5 d.p.c. due primarily to vascular and cardiac abnormalities. Analyses of 9.5 d.p.c. Eng-/- yolk sacs (YS) revealed abnormal blood vessels as well as anemia. It is not clear whether the latter results from an indirect effect due to insufficient blood flow, or a direct effect of endoglin on blood development. Here we addressed this question by investigating the role of this receptor *in vivo* during early hematopoiesis. Analysis of YS from E9.5 Eng-/- embryos show significantly reduced numbers of hematopoietic colony-forming cells (CFCs), in particular GEMMs and BFU-Es, when compared to both wild-type and heterozygous embryos. This is accompanied by decreased expression of important hematopoietic genes, including Embryonic Globin, Gata-1, and Scl. Based on these findings we set up experiments to characterize the nature and function of endoglin-expressing cells in wild-type mice. Remarkably, hematopoietic activity was restricted to the Eng+ cell fraction at every time point (from E7.5 to E9.5) studied. Eng- cells failed to produce any hematopoietic colonies. Subsequently, we subfractionated these E7.5-9.5 cells into four distinctive cell fractions based on Eng and Flk-1 expression. The vast majority of colony-forming cells at E7.5 reside within the Eng+ Flk-1+ fraction. This pattern changes as the embryo develops, and at E9.5, most of the hematopoietic activity resides within the Eng+ Flk-1- population. Transcriptional profiling of the 4 fractions in E7.5 embryos reveals that the Eng+Flk-1+ fraction highly expresses endothelial and hematopoietic markers, such as Fli-1, VE-Cadherin, Tie-2, CD31, CD34, Gata-1, Gata-2, Scl, and Lmo2. Moreover, ligands, receptors type I and II, and target genes of the TGF-beta superfamily were found highly and distinctively expressed in these fractions, suggesting that endoglin plays a critical role in these early signaling events. Taken together, our data demonstrate that endoglin is not only necessary for proper hematopoiesis, but also identifies the first wave of hematopoietic progenitors during early development, pointing to a defined role for endoglin and the TGF-β superfamily signaling in early hematopoiesis.

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Poster Board Number: 2388

EMERGENCE OF EMP-DERIVED DEFINITIVE HEMATOPOIESIS IN THE MAMMALIAN EMBRYO AND EMBRYONIC STEM CELLS

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The ontogeny of hematopoiesis in mammalian embryos is complicated by the requirement for functional blood cells prior to the emergence of hematopoietic stem cells (HSC) or the bone marrow microenvironment. In the murine embryo, transplantable HSC are first evident at embryonic day (E) 10.5 and small numbers begin to seed the fetal liver between E11.5 and E12.5. However, two overlapping waves of hematopoietic potential arise in the yolk sac before E10.5. The first "primitive" wave produces progenitors from E7.25 to E8.5 with primitive erythroid, megakaryocyte and macrophage lineage potential. At E8.5, a second wave of hematopoiesis begins in the yolk sac and generates definitive erythroid, megakaryocyte, and multiple myeloid lineage potential that is the proposed source of hematopoietic progenitors that seed the fetal liver before HSC colonization. A similar pattern of pre-HSC hematopoiesis has been found in human and as well as zebrafish embryos, where this first transient definitive wave has been termed "erythromyeloid progenitors" or EMP. We have determined that EMP in the E9.5 mouse yolk sac have a unique immunophenotype, co-expressing ckit, CD41, CD16/32 and endoglin. Other markers associated with HSC formation (AA4.1, ScaI) or with lymphoid potential (IL7R, Flt3) are not present on these cells at E9.5 while, as seen in HSC emergence, the "endothelial" markers VE-Cadherin and Flk1 are present. This immunophenotypically-defined EMP population rapidly forms definitive erythroid, megakaryocyte, macrophage, and all forms of granulocytic cells *in vitro* and contains all of the definitive hematopoietic colony-forming cells present in the E9.5 yolk sac. Consistent with the lack of lymphoid markers, EMP do not generate (CD19+B220+) B-cells *in vitro*, in contrast to bone marrow-derived Lin-/ckit+/ScaI- cells. Like CMP in the marrow, single EMP can generate both erythroid and granulocyte potential. The differentiation of embryonic stem cells (ES) and induced pluripotent stem cells (iPS) cells into mature cells types offers the hope of cell-based therapies. Analysis of differentiating murine ES cells reveals overlapping waves of primitive and definitive hematopoietic colony forming potential. We demonstrate this definitive potential resides in an EMP-like (ckit+/CD41+/FcGR+) population. Our studies support the concept that blood cell emergence during ES cell differentiation closely mimics pre-HSC hematopoiesis in the yolk sac. Though these EMP were enriched at E9.5 in the yolk sac, they are also found at low levels in the fetal blood, embryo proper and placenta, consistent with their entrance into the circulation. By E10.5, EMP were most highly enriched in the newly formed fetal liver. Additionally by E12.5, a time when the first few HSCs are detected in the fetal liver, we find active erythropoiesis and granulopoiesis in the liver and the first definitive red blood cells and neutrophils in the bloodstream. Therefore, we believe the yolk sac definitive progenitors' fate is to populate the fetal liver and thus provide the first definitive erythrocytes and granulocytes for the embryo.

Poster Board Number: 2390

LOSS OF CSL-DEPENDENT NOTCH SIGNALING IN THE HEMATOPOIETIC MICROENVIRONMENT LEADS TO A LETHAL MYELOPROLIFERATIVE DISORDER

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In the adult, hematopoietic stem cell (HSC) homeostasis is regulated by specialized niches in the bone marrow (BM). The three-dimensional structure of the BM microenvironment includes the extracellular matrix and different cell types, such as fibroblasts, adipocytes, osteoblasts, endothelial and mesenchymal stem cells (MSC). While the role of the osteoblastic niche has been extensively studied, the instructive role of other cell types in providing homeostatic stimuli to resident HSC and multipotential progenitors needs to be further explored. Several molecular effectors have been implicated in the fine-tuned regulation of HSC functions by the BM niches, including Notch signaling and its ligands. Importantly, Notch signaling plays a critical role on hematopoiesis, endothelial cell biology, osteoblasts homeostasis and MSC differentiation. Although the cell-autonomous role of Notch signaling on hematopoietic cells has been extensively studied, to date, no studies have formally demonstrated whether disruption of Notch signaling on the hematopoietic microenvironment has an impact on hematopoietic homeostasis. To investigate the role of Notch signaling in the BM microenvironment, we used the RBP-Jlox/loxMx1Cre mice model. Deletion of the DNA-binding motif of RBP-J by Cre results in the loss of CSL-dependent signaling from all Notch receptors. The Mx1 promoter drives Cre expression not only on hematopoietic cells but also on the stromal component of the hematopoietic organs. Effective deletion of the RBP-J exon was detected by PCR on hematopoietic cells, as well as on sorted BM osteoblasts, MSC and endothelial progenitor cells (EPC) of RBP-Jlox/lox Cre+ mice following induction by Poly(I)-poly(C) (pIpC). To determine the effects of Notch-defective BM stroma on hematopoiesis, we transplanted normal BM from CD45.1 B6/BoyJ mice into lethally irradiated CD45.2 RBP-Jlox/loxMxCre+ (RBP-J KO) or RBP-Jlox/loxMxCre- (Co). At wk 8 from transplant, both groups were induced with pIpC. At day 50 to 70 from induction, all RBP-J KO recipients showed increased WBC, in particular neutrophils (KO 11.75±6.63 vs Co 5.29±1.86 k/uL) and increased Gr1+/Mac1+ cells in the bone marrow (KO 49.10%±4.83%, vs Co 33.93%±1.90%) spleen (KO 2.88%±0.97% vs Co 1.00%±0.34%) and peripheral blood (KO 19.27%±4.19% vs Co 7.57%±1.76%), increased circulating HSC (KO 0.86%±0.32% vs Co 0.25%±0.12%), and splenomegaly, suggesting a myeloproliferative disorder. Survival at 5 months was 12% for the RBPJ KO mice compared to 100% for the control mice. Histological analysis of BM and spleen confirmed a MPD-like disorder and showed increased microvascular density and increased CD31+ EC by IHC. Also, higher frequency of the CD45-CD105+CD31+ cell subset, enriched in EC, was seen in the BM and spleen of RBP-J KO mice. Transplantation of BM cells from RBP-J KO mice with advanced disease did not transfer the myeloproliferative disease to secondary WT recipients. Transplantation of RBP-J KO BM HSC into WT mice resulted in decreased T-cells; increased B-cells, as previously described, but did not induce any obvious defect in myelopoiesis. Taken together, these data suggest that the alteration of the hematopoietic system in floxed RBP-J mice is driven by the microenvironment. Further studies are ongoing to identify whether the endothelium or other cellular types are involved in mediating the non-cell autonomous myeloproliferative disease.

Poster Board Number: 2392

SQSTM1 (P62) EXTRINSICALLY REGULATES HEMATOPOIETIC STEM CELL AND PROGENITOR MOBILIZATION

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Hematopoietic stem cell and progenitor (HSC/P) mobilization is a complex process that involves interaction of HSC/P with hematopoietic microenvironment. The molecular signatures implicated in the control of the hematopoietic microenvironment on HSC/P mobilization and retention remain poorly understood. Using loss of function chimeric mice models, we report cell-nonautonomous requirement of SQSTM1 (p62) in adult HSC/P mobilization. We demonstrate that deficiency of p62 in the hematopoietic microen-

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vironment, but not in HSC/P, induces HSC/P egress. Increased circulation of HSC/P was associated with reduction in trabecular bone volume and architecture. Interestingly, additional deletion of Nbr1, another member in phox bemp1 (PB1) domain-containing proteins, in a p62-deficient background (p62-/-, Nbr1Δ/Δ) rescued HSC/P mobilization and reverted bone architecture *in vivo*. Combined deficiency of p62 and Nbr1 also induced expansion of bone marrow mesenchymal progenitor pool and altered osteogenic differentiation. However, loss of Erk1, which is negatively regulated by p62 to inhibit adipogenesis, in p62-deficient background (p62-/-; Erk1-/-) did not rescue increased HSC/P mobilization *in vivo*, suggesting HSC/P mobilization was independent of adipogenesis. Collectively, our studies provide genetic evidence that p62 and Nbr1 play cell-nonautonomous epistatic antagonism in bone remodeling in mammalian HSC/P mobilization *in vivo*.

Poster Board Number: 2394

HEMATOPOIETIC STEM CELL ACTIVITY AND POLARITY ARE CONSERVED IN THE ABSENCE OF ATYPICAL PKCζ AND λ

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Stem cell pool is considered to be maintained by a balance between symmetric and asymmetric division of stem cells. Cell polarity model proposes that the facultative use of symmetric and asymmetric cell division is orchestrated by a polarity complex consisting of partitioning-defective proteins: Par3, Par6 and atypical protein kinase C (aPKCζ and aPKCλ) which regulates planar symmetry of dividing stem cells with respect to the signaling microenvironment. However, the role of the polarity complex has been unexplored in mammalian adult stem cell maintenance. Here we report that, in contrast to accepted paradigms, activity of adult hematopoietic stem cell (HSC) does not depend on the polarity complex-determining kinases: aPKCζ or aPKCλ or both *in vivo*. Mice, having constitutive and hematopoietic-specific (Vav1-Cre) deletion of aPKCζ and aPKCλ, respectively, are phenotypically normal and their litters follow Mendelian ratio of inheritance. Inducible genetic inactivation of aPKCζ and aPKCλ in HSC does not affect HSC polarization, self-renewal, engraftment, differentiation, steady state or G-CSF-induced mobilization and interaction within the bone marrow microenvironment either. In addition, aPKCζ and aPKCλ-deficient HSCs elicited a normal pattern of hematopoietic recovery secondary to myeloablative stress. Taken together, expression of aPKCζ or aPKCλ or both are dispensable for primitive and adult HSC fate determination, in steady state and stressed hematopoiesis; jeopardizing the dogma of evolutionary conserved aPKCζ/λ-directed cell polarity in mammalian HSC fate determination and suggesting that alternative kinases can replace aPKCζ/λ in the composition of the polarity complex.

Poster Board Number: 2396

EMBRYONIC STEM CELL-DERIVED FACTORS INHIBIT T EFFECTOR ACTIVATION AND INDUCE T REGULATORY CELLS BY MODULATING PKC-Θ ACTIVATION.

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Embryonic stem cells (ESC) possess immune privileged properties and have the capacity to modulate immune activation. However, the mechanisms by which ESC inhibit immune activation remain mostly unknown. We have previously shown that ESC derived factors block dendritic cell maturation and thereby affect T cell activation indirectly. Our ongoing studies have elucidated that ESC derived factors also have a direct effect on T cell activation. In mixed lymphocyte reaction assays, ESC derived factors significantly down-regulated IL-2 and IFN-γ expression, while up-regulating IL-10 and TGF-β expression. Also, ESC derived factors suppressed the expression of Th1 transcription factor Tbet, while enhancing the expression of Treg transcription factor Foxp3. Furthermore, PBMC/purified T cells activated with CD3/CD28, ConA and PMA proliferated poorly in the presence of ESC derived factors, while proliferation in response to ionomycin was not affected. Western blot analysis indicated that ESC derived factors prevented PKC-θ phosphorylation without influencing total PKC-θ levels. Moreover, IκB-α degradation was abrogated, confirming absence of PKC-θ activity. Incubation of ESC derived factors with recombinant GST-PKC-θ resulted in the pull-down of four proteins. The purified proteins are currently being analyzed by mass spectrometry. In conclusion, ESC's are able to directly impact T cell activation and polarization, likely by negatively regulating PKC-θ through potentially novel factors.

Poster Board Number: 2398

CONTROLLED GENERATION OF T CELLS FROM HEMATOPOIETIC PROGENITOR CELLS (HPC) DERIVED FROM PLURIPOTENT CELL SOURCES (PSC) FOR HIV TRANSPLANTATION THERAPY

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Embryonic and induced PSC hold great potential for cell-based therapies due to their ability to differentiate into all cell types, including blood cells. Blood cells are the principal target of many infectious diseases, including human immunodeficiency virus (HIV). HIV propagates by debilitating the immune system; specifically CD4+ T cells that mediate resistance to opportunistic infections. Developing better technologies to generate T cells from PSC, either in their normal state or mutated to be more resistant to HIV infection could provide important tools to gain new insight into this disease. Results will be presented comparing the differentiation kinetics of ESCs and iPSCs derived from mature and immature T cell subsets (T-iPSCs) to HPCs in a serum-free assay with defined aggregate sizes. Additionally, novel micropatterning techniques for immobilizing Delta-like 1 (DL1) ligand are being developed; these will be incorporated into T-cell assays to characterize T cell development under defined conditions. Finally, human blood cell targets are being compared for their T-cell generation properties. Controlled generation of HIV-resistant T cells from HPCs can lead to the production of cell-based vac-

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cines on a large scale that will provide a co-ordinated immunological defense against HIV progression.

Poster Board Number: 2400

TRANSGENE FREE DISEASE DERIVED-IPS DIFFERENTIATION INTO HEMATOPOIETIC AND ERYTHROID CELLS

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Human induced pluripotent stem cells (hiPSC) have been shown to have differentiation potential similar to human embryonic stem cells (hESC). In particular, the questions of whether the source and the age of the somatic cells used to produce iPS have an influence on the differentiation potential of the iPS, has not been fully explored. From our previously study shown that co-culture hESC with immortalized fetal hepatocytes (FHB) yield CD34+ cells that can be further expanded in serum-free liquid culture to obtain large number of megaloblastic nucleated erythroblasts. With extending the liquid culture, we found that the hemoglobin composition switching from basophilic erythroblasts expressing Hb Gower I (22) to orthochromatic erythroblast hemoglobin Gower II(22). By extending the co-culture of hESC with FHB from 14 day to 35 day, the CD34+ obtained from extending culture follow expanding and differentiating by liquid culture, shown more developmentally mature fetal-liver like erythroblast which expressed HbF (22). In the current study, we have used retroviruses expressing Oct4, Sox2, Klf4 and c-Myc to produce iPS from embryonic and fetal mesenchymal cells, from adult skin fibroblasts, and hair keratinocytes, and compared them in details with hESC. Morphology, growth characteristics, surface antigen expression and mRNA expression (Affymetrix Human Gene 1.0 ST Array) were undistinguishable from undifferentiated H1 and H9 hESC. PCR analysis of pluripotency factors revealed that in most iPS clones the four exogenous reprogramming factors were mostly silenced except in one interesting clone, obtained from fetal liver which had not silenced any of the four factors. Differentiation along the hematopoietic and erythroid lineages yielded numbers of CD34+ cells (2 to 10%) similar to those obtained with hESCs and red blood cells that undergo the same two globin switches as hESCs. Interestingly, the iPS clone that did not silenced the four factors differentiated predominantly into CD34+ cells, yielding 10 to 20 times more CD34+ cells than the other iPS, although these CD34+ cells had very poor erythroid differentiation potential. We conclude that iPS with apparently normal globin switching potential can be obtained from embryonic, fetal or adult somatic cells originating from multiple tissue sources. Aberrantly reprogrammed iPS with interesting differentiation potential are also obtained. Aberrant reprogramming can be associated with poor silencing of the reprogramming factors. In order to avoid the consequences caused by virus insertion, we reprogrammed disease fibroblasts with episome vectors. The iPS cells from alpha thalassemia fibroblast that has four alpha gene deletions (FIL-/SEA-), and the aTiPS expressed typical hESC markers. The aTiPS can form teratomas with injected into immuno-compromise mice, and shown no trasngene insertions in the PCR results. The CD34+ cells obtain from 14 days coculture of aTiPS with FHB, after expend and maturated by liquid culture, express Gower I (22) compare to the erythroblast from the hESC and other iPS from normal cells with the same 14 co-culture protocol that express Gower II (22). However, the erythroblast derived from aTiPS didn't show microcytic or hypochromic features in our 14 days differentiation.

Poster Board Number: 2402

DEFINING OPTIMAL CONDITIONS FOR GENERATING CELLS OF THE HAEMATOPOIETIC LINEAGE FROM INDUCED PLURIPOTENT STEM CELLS DERIVED FROM DOWN SYNDROME FIBROBLASTS

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Down Syndrome (DS) children are prone to develop blood abnormalities. The blood disorder starts in the fetus, presenting as Transient Abnormal Myelopoiesis (TAM) in neonates which, in some cases, evolves into leukaemia, particularly Acute Megarkaryoblastic leukaemia (AMKL). In this study we aim to use iPS cells made from DS fibroblasts as a novel way to recreate the abnormal haematopoiesis *in vitro*. Stable iPS cell lines have been created from normal and DS fibroblasts using two non-viral episomal vectors containing six reprogramming factors-OCT4, Sox2, NANOG, LIN28, c-Myc and KLF4. Both normal and DS iPS cell clones are free of episomal vector DNA and episome-derived transcripts. Cytogenetic studies confirmed the presence of trisomy 21 and gene expression studies showed the expression of pluripotency associated genes. The expression of a selection of these genes including SSEA-4 and TRA-1-60 was verified by flow cytometry and immunofluorescent staining. These iPS cells also have the ability to differentiate into all three germ layers and develop teratomas in mice. Embryoid bodies (EBs) formed from these iPS cells were subjected to two different haematopoietic differentiation methods. The first is based on the co-culture of EBs on OP9 cells and growth factors (SCF, Flt3 ligand, FGF, TPO, BMP4, VEGF) for two weeks. The other method involved a 20-day culture of EBs on low attachment plates in the presence of high dose growth factors (SCF, Flt3 ligand, TPO, BMP4, VEGF). Both methods were able to generate CD34 and CD45+ cells detected by flow cytometry yielding 4-9% CD34/CD45+ double positive cells. We have modified the culture method to increase the yield. The haematopoietic cells generated are isolated by MACs and further characterised using haematopoietic progenitor assays to verify the abnormal differentiation and proliferation characteristics of DS iPS cells likely associated with the evolution of DS into leukaemia reported in *in vivo* studies. Gene expression profiling of isolated progenitors aiming to identify abnormal gene expressions linked to the aberrant haematopoiesis of DS iPS cells will be discussed.

Poster Board Number: 2404

OSTEOBLASTS DERIVED FROM MSC PROMOTE CELL CYCLE ENTRY OF CD34+ CELLS AND EXPANSION OF HEMATOPOIETIC PROGENITORS

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Introduction: Expansion of Umbilical cord blood (UCB) hematopoietic cells is of high interest to broaden their use in transplantation medicine. Indeed, UCB transplantation in adults is currently limited by the small amount of hematopoietic progenitor cells (HPCs) present in each unit. In this work, we aimed to increase the expansion of HPCs and megakaryocyte progenitor cell (MPC) by the combine use of optimized cytokine cocktails and feeder cells

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normally present within the Bone marrow (BM) or UCB environment. Material & Methods: Osteoblasts (OST) derived from BM Mesenchymal Stem Cells (MSC) and Human umbilical vein endothelial cells (HUVECs) were tested as feeder cells for CD34+ UCB cells. The cultures were complemented with the cytokine cocktails OMPC (TPO, SCF, FLT-3) and BS1 (TPO, SCF, IL-6, IL-9) used for MPC expansion (day 0-4) and megakaryocyte differentiation (day 4-14). 4 co-culture conditions were tested; OST with (OST C) or without contact in trans-wells (OST WC) and, HUVECs with (HUVEC C) or without contact (HUVEC WC). The control conditions consisted of CD34+ cells grown in 24-well (CTL C) and in trans-wells (control for non-contact condition (CTL WC)). HPCs (CFC) and MPCs (CFU-MK) frequencies were measured using standard progenitor assays. 4 independent experiments were done for all tests. Results: First, the impact of co-culture with the HUVEC or OST feeder cells on the proliferation of UCB CD34+ cells was assessed. Total cell expansion in OST C cultures was increased by 1.6-fold and 1.2-fold compared to the CTL C at days 6 and 14, respectively. There was no differences in the frequencies of CD34+ cells between the different co-culture conditions, though the former tended to be greater in the co-cultures with OST C at day-6 and OST WC at day-14 compared to the controls (2- and 3-fold greater, respectively). Next, the impact of the feeder cells on HPC expansions were investigated by colony assays. Co-culture with OST WC and HUVEC WC increased the expansion of myeloid HPC by 1.7 ($p<0.05$) and 3.7-fold ($p>0.05$), and of MPC by 3.2- ($p<0.01$) and 2.5-fold ($p<0.1$) compared to the CTL WC at day 6, respectively. The increased expansion of hematopoietic cells and HPCs obtained in some co-culture conditions suggest that the feeder cells may have a positive impact on the proliferation and/or cell cycle entry. To investigate this, cell cycle and cell cycle entry of quiescent hematopoietic cells were analysed by cytometry (Ki-67 and 7AAD). The proportion of CD34+ cells still in G0 in HUVEC C cultures (16.3±5.0) were 5.6- ($p<0.07$) and 5.2-fold ($p<0.04$) greater than those in the OST C (2.9±1.3) and CTL C (3.1±0.8) cultures at day 4. Similar observations were made for the total nucleated cell population. In contrast, hematopoietic cells co-cultured with OST (C and WC) generally entered the G1 phase faster than those present in the control (74 ± 3 vs. 65 ± 6 $p<0.15$ for contact, and 72 ± 8 vs. 63 ± 8, $p< 0.01$ for non contact) and HUVEC C (61±7, $p<0.07$) cultures. Conclusion: These results demonstrate that expansion of myeloid and megakaryocyte progenitors are significantly increased when CD34+ cells are co-cultured with OST without contact. The results also suggest that growth factors released by OST stimulate the cell cycle entry of quiescent CD34+ cells, while direct contact with HUVEC has the opposite effect. Hence, co-culture of HPCs with OST in the presence of optimized cytokine cocktails provides an interesting strategy to increase the amount of HPCs and MPCs available for transplantation.

Poster Board Number: 2406

THE ROLE OF RETINOIC ACID SIGNALING IN PRIMITIVE HEMATOPOIESIS

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The emergence of the primitive erythroid lineage in the blood islands of the yolk sac defines the onset of hematopoiesis in the mammalian embryo. The primitive erythroid lineage is unique within the hematopoietic system in that it represents a transient developmental program that is restricted to the yolk. In addition to primitive erythrocytes, the early yolk sac also generates macrophages, megakaryocytes and progenitors of the definitive erythroid lineage. The transient and restricted nature of primitive erythropoiesis suggests that both its onset and termination are tightly controlled. Insights into the regulation of this early blood cell lineage have been provided by studies in the early embryo and the embryonic stem cell (ESC) differentiation model, which have shown that primitive erythropoiesis is controlled in part by the VEGF/Flk-1, Wnt, Notch and activin pathways. Findings from recent studies suggest that retinoic acid (RA) signaling may also play a role in the regulation of this early hematopoietic program. When added to ES cell differentiation cultures, RA was found to inhibit primitive hematopoiesis. RA exerts its effect through three different retinoic acid receptors (RARs) alpha (α), beta (β), and gamma (γ). To determine if the inhibitory effect is mediated specifically through one of these receptors, we treated whole mouse ES cell-derived embryoid body (EB) populations or Flk-1+ progenitors isolated from them with α (RAR-α) or γ (RAR-γ) specific agonists. When added at the onset of primitive erythropoiesis, RAR-α but not RAR-γ completely inhibited the generation of the primitive erythroid and macrophage lineages, characteristic of the *in vitro* equivalent of yolk sac hematopoiesis. All trans RA also inhibited this early stage of hematopoiesis. The addition of the RAR-α antagonist together with RAR-α or RA partially rescued the block in primitive hematopoietic development, indicating that the inhibitory effect of RA is mediated through the α receptor. The differential effects of RAR-α and RAR-γ on the ESC-derived primitive hematopoietic program provide a unique model for further dissecting the role of this signaling pathway in hematopoietic specification.

Poster Board Number: 2408

EFFECTS OF CORD BLOOD NK CELLS ON THE HOMING OF CORD BLOOD STEM CELLS

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Stem cell (SC) transplantation is a common treatment for haematological malignancies and bone marrow (BM) failure. Mobilized peripheral blood stem cells and BM are the most common sources for haematopoietic stem cell transplantation (HSCT). Currently, umbilical cord blood stem cells (CBSC) are also being used. CBSC have many attractive properties such as low incidence and severity of graft-versus-host disease (GvHD), a strong graft-versus-leukemia (GvL) and an increased tolerance to HLA mismatch are highly relevant. However, there are also disadvantages; delayed recovery and post-transplant infections have the most important clinical considerations. Previous studies have reported that accessory cells, such as CD8, regulatory T cells (Tregs) and Natural Killer (NK) cells have a positive effect on SC engraftment after transplantation. On this basis, we decided to analyse the effect of accessory cells: NK cells, CD4+ and CD8+ on engraftment of CBSC. Using a humanized model, Rag2-/-IL-2Rgc-/- mice, we analyzed 4 different groups. The first group of mice were transplanted with CD34+ cells only, the second, third and fourth group received NK cells, CD4+ cells and CD8+ cells, respectively, in addition to the dose of CD34+ cells. After 5 weeks we analyzed engraftment based on the presence of CD45+ cells within the BM. We observed a higher level of engraftment in the group of mice that received SC plus NK cells compared to the other groups that received CD4+ and CD8+. Based on these results we decided to study the effect that NK cells have on the migration of CBSC, and the mechanisms of interaction between these two cells. We first characterised different homing receptors such as alpha and beta integrins, P-selectin receptor, CD47 and CXCR4. This characterisation was done on resting freshly isolated CBSC and NK cells and also on CBSC co-cultured with NK cells. Additionally, in order to analyse the migration capacity of CBSC in the presence of NK cells we performed transwell migration assays with CBSC alone and in the presence of NK cells, using SDF1a as a stimulus for migration at a concentration of 125ng/mL. Our results so far suggest that NK cells seem to play an important role in CBSC migration and engraftment. However we still need to propose a possible mechanism by which NK cells have an effect on CBSC migration.

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Poster Board Number: 2410

EVALUATION OF SEVEN DAY COLONY FORMING UNIT (CFU) ASSAY FOR THE ASSESSMENT OF CORD BLOOD (CB) POTENCY AND HEMATOPOIETIC PRECURSOR CELL (HPC) CONTENT

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Background: Choosing the perfect cord blood unit (CBU) for transplantation is based on tissue typing and the stem cell potential of the unit. CBUs are routinely assessed at collection with regards to total nucleated cells and CD34+cell count. The potency of the unit to be released is assessed post-thaw by the 14-day CFU (colony forming units) assay. This assay has a disadvantage in being time-consuming, and an alternative assay with a shorter 7-day incubation period has been proposed (MethoCult® Express, Stem Cell technologies). Aim: To compare the performance of the 7-day CFU assay with the traditional 14-day CFU assay, using our standard CFU protocol with MethoCult® GF medium (7d and 14d-assay) and MethoCult® Express (7d -assay). Materials and Methods: CBUs (n=58) were evaluated for CFU growth and colony number post-cryopreservation. Each unit was cultured in MethoCult® GF with erythropoietin or MethoCult® Express (StemCell technologies) for 7 and 14 days respectively. The total number of colonies was counted manually. Results and Discussion: All CBUs showed colony growth in both assays. Colonies were smaller with the 7d-assay, and it was not possible to discriminate between different cell colony types. The CFU counts of the 7d and 14d assays correlated well, irrespective of which growth medium that was used. MethoCult® GF 7d vs 14d: R2=0,87 and MethoCult® Express 7d vs MethoCult® GF 14d: R2=0,89. Furthermore, the CFU count of the 7d MethoCult® GF and the 7d MethoCult® Express also correlated very well, R2=0,94. In summary, the 7-day assay is useful when results are needed fast, however using the more expensive culture medium MethoCult® Express does not seem to be necessary, as very similar results were obtained using the standard MethoCult® GF medium.

Poster Board Number: 2412

MICRORNA-126 MODULATES THE SENSITIVITY OF HEMATOPOIETIC STEM AND PROGENITOR CELLS TO ENVIRONMENTAL SIGNALS

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We recently identified miR-126 as a hematopoietic stem cell (HSC) microRNA. Yet, little is known about its function in hematopoietic stem and progenitor cells (HSPC). We generated a stable miR-126 knockdown (kd) or forced its expression ("knock-in", ki) in mouse HSPC using lentiviral vectors. Kd or ki cells were competitively transplanted with congenic, control vector-transduced cells, and hematopoietic chimerism was followed for >1 year in primary and secondary recipients. Forced expression of miR-126 resulted in a higher myeloid and B cell contribution compared to control cells at week 2-4 after transplant. However, this was followed by a progressively decreasing contribution of miR-126 ki cells to all hematopoietic lineages and a nearly complete depletion of Kit+Sca+Lin- (KSL) BM cells by 6 weeks after transplant. At 3 weeks post-transplant, when miR-126 ki KSL cells could still be detected, we found an increased proliferative index in these cells as judged by *in vivo* EdU incorporation. These data suggest that miR-126 ki might favor HSC commitment at the cost of self-renewal. This phenotype was specific for miR-126 and not due to vector toxicity, as we demonstrate stable, long term overexpression of several control miRNAs *in vivo*. Moreover, miR-126 ki cells showed normal clonogenic activity *in vitro*. On the contrary, miR-126 kd HSPC engrafted long-term in competitive transplantation assays, and miR-126kd cell chimerism was >2 fold above control specifically in the HSC-enriched Lin-Kit+Sca+CD150+CD48- bone marrow (BM) compartment. We furthermore noted enhanced myeloid and/or lymphoid contribution during the early phases of reconstitution which was reproducible upon serial transplantation, suggesting that miR-126 kd enhances hematopoiesis particularly under stress conditions, possibly by modulating HSC responses to environmental signals. We then optimized a protocol to stably knock down miR-126 in human cord blood (huCB) HSPC, and validated this approach by demonstrating upregulation of previously described miR-126 targets including the beta subunit of phosphoinositide-3-kinase (Pi3K). Importantly, we provide evidence for increased activation of the Pi3K-Akt pathway in miR-126 kd huCB cells upon cytokine stimulation, and these cells show enhanced growth and increased CFU-C content when cultured in the presence of early acting cytokines. Identification of additional miR-126 targets is ongoing using unbiased proteomic and transcriptomic approaches. In summary, these data identify miR-126 as a novel HSC regulator, possibly modulating the responsiveness of HSPC to growth/survival & differentiation signals. Further study of miR-126 will provide new insights into hematopoietic stem cell biology which might facilitate the usage of these clinically relevant cells for novel gene therapy approaches.

Poster Board Number: 2414

CAN AMNIOTIC FLUID STEM CELLS BE AN OPTION FOR AUTOLOGOUS CELL BASED GENE THERAPY *IN UTERO*?

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Introduction: Prenatal stem cell-based gene therapy using transduced amniotic fluid stem (AFS) cells is a possible strategy to treat congenital diseases. Allogeneic cells can be rejected in immune-competent animals, even in fetal life. Autologous cell therapy offers an alternative option. Mouse amniotic fluid (AF) contains ~1% c-Kit(+)/Lin(-) AFS cells, that can differentiate into the three germ layers. We compared congenic (autologous) versus allogenic *in utero* transplantation of AFS cells in mice. Materials and Method: Amniotic fluid was collected from yellow fluorescence protein YFP(+) C57BL/6 mice at E13.5. c-Kit(+)/Lin(-) cells were injected into the peritoneal cavity of every fetus from C57BL/6 (congenic transplantation) or CD1 wild type (allogenic transplantation) dams on E13.5. Engraftment was assessed at 1 and 3 months of age. Results: Twelve out of 20 (60%) CD1 and 4 out of 7 (57%) injected C57BL/6 pups survived to term. There was significantly higher peripheral blood engraftment in congenic (5.15±1.40%), compared with allogenic (1.65±0.60%, $p<0.05$) models by flow cytometry at 1 months of age. Colony-forming cell (CFC) assays were performed by plating the bone marrow Lin(-) cells of transplanted animals into methylcellulose semi-solid medium (MethoCult, StemCell Technologies). All types of the hematopoietic CFC were observed after 14 days incubation. Fluorescence microscopy showed small population of yellow colonies in the bone marrow of congenic but not allogenic injected mice. A positive YFP bands were detected by PCR in the liver, spleen, adrenal glands of recipient congenic mice, while only in the liver of allogenic one. Immunohistochemistry further confirmed the YFP cells engrafting in the liver of both arms at 3 month-old. Conclusion: These initial studies confirm that autologous cell therapy may give better engraftment than allogenic.

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Poster Board Number: 2416

SHEEP AMNIOTIC FLUID DERIVED CD34+ STEM CELLS COULD ENGRAFT AND RESTORE THE HEMATOPOIETIC SYSTEM OF NSG MICE

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Introduction: We have previously shown that human and mouse amniotic fluid (AF) c-Kit(+)/Lin(-) amniotic fluid stem (AFS) cells, displayed hematopoietic potential both *in vitro* and *in vivo*. Currently, there is no c-Kit antibody available for sheep cells, and the human antibody does not cross react with sheep. Recently a novel CD34 monoclonal antibody specific to sheep has been developed. In this study, we tested whether CD34+ sheep AFS cells have functional haematopoietic potential. Materials and Method: Sheep amniotic fluid was collected by ultrasound guided amniocentesis at 59.5±4.5 days (term=145 days). A novel sheep specific CD34 monoclonal antibody was used to select cells in a microbeads magnetic sorting system. Sheep AFS CD34 + cells were cultured with StemSpan SFEM medium plus cytokine cocktail, and transduced using an HIV vector encoding enhanced green fluorescent protein (GFP, MOI=50) for 24 hours. Transduced AFS CD34+ cells (3x10^5 cells in 200µl PBS) were injected intravenously into NOD-scid gamma (NSG) mice (N=10). Engraftment of transduced cells was assessed after 3 months. Results: Sheep CD34+ AFS cells formed colonies using methylcellulose-based assay, and were positive for CD45, CD133, but negative for CD14, CD31, CD44, CD58 and CD166. 80% injected NSG mice survived up to 3 months. Flow cytometric analysis at 3 months showed GFP positive cells in the peripheral blood (3.38±0.92%) and bone marrow (12.9±1.6%). The liver, spleen, bone marrow and adrenal gland were positive for GFP DNA by PCR. Immunohistochemistry confirmed the engraftment of donor cells in liver. Conclusion: This study demonstrates the hematopoietic potential of CD34+ sheep AFS cells. These cells could be applied as an autologous transplantation resource into large animal models of disease and could correspond to early haematopoietic progenitors identified previously in mouse and human AF.

MESENCHYMAL CELL LINEAGE ANALYSIS

Poster Board Number: 2418

HUMAN MENSTRUAL BLOOD DERIVED MESENCHYMAL CELLS EFFICIENTLY MANAGE OXIDATIVE STRESS

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Mesenchymal stem cells transplantation (MSCs) proves to be useful to treat diseases in which tissue damage is linked to oxidative stress (OS). Recently, it has been demonstrated that stem cells can be isolated from menstrual blood. This is an important new source of stem cells, since they can be easily obtained, do not require invasive procedures, and availability is virtually endless. Thus, the aim of this work was to isolate, characterize, differentiate menstrual blood-derived stem cells (MBMC) and evaluate whether MBMC can manage oxidative stress (OS). Human menstrual blood was collected from eleven healthy female subjects when menstrual flow initiated. Mononuclear cells were obtained by Ficoll-Paque gradient and plated in culture dishes. Human menstrual blood-derived cells presented a subpopulation of adherent cells with spindle-shape morphology that were highly proliferative. Population doubling time was 38.07 ± 1.9 hours of women not using contraception while MBMC from women who use contraceptives double their population every 35.97 ± 1.7 hours, with no statistical difference between samples. After five passages, the presence of surface markers was evaluated by flow cytometry. These cells were positive for the mesenchymal cell markers CD90 (93.3%), CD73 (85.5%); and negative for the hematopoietic markers CD34, CD45, CD14, CD19 and HLA-DR, the endothelial marker CD31, CD133 and the stem cell marker c-kit. This immunophenotypic profile was maintained after fifteen passages. The expression of pluripotency molecules was evaluated by RT-PCR and immunofluorescence. At the mRNA level, menstrual blood-derived cells expressed the core embryonic stem (ES) cell regulators Oct4, Sox2, nanog and Klf4. At the protein level, these cells stained positive for the pluripotency markers SSEA-4, TRA1-60 and TRA1-81. Moreover, when adipogenic and osteogenic differentiation was induced, these cells formed fat vacuoles demonstrated by Oil Red O staining and showed calcium deposits when stained with Alizarin Red, respectively. MBMC were cultured with increasing concentrations of reactive oxygen species (ROS; source: H2O2) and its viability was evaluated by staining of adherent cells with crystal violet. These cells showed high resistance to OS-induced death (IC50: 1.8mM). Moreover, MBMC are capable of producing large quantities of total H2O2 (90.6 ± 9.7 nmol H2O2 /h/mg of protein) and NADPHoxidase is responsible for one third of this production. In conclusion, menstrual blood-derived cells are highly proliferative, express mesenchymal stem cell surface markers and differentiate into osteocytes and adipocytes *in vitro*, indicating that they are multipotent mesenchymal cells. These cells express pluripotency markers, suggesting that they are a powerful source of progenitors and could potentially be differentiated in other mesodermal tissue types. Moreover, these cells presented high resistance to OS and might be considered a tool for cell therapy strategy aimed to treat patients with diseases in which onset and progression is associated with OS.

Poster Board Number: 2420

TRANSPLANTATION AND BIODISTRIBUTION OF HUMAN PLACENTA-DERIVED MESENCHYMAL STROMAL CELLS IN IMMUNOCOMPETENT MICE SUBMITTED TO MYOCARDIAL INFARCTION.

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Death of cardiomyocytes due to myocardial infarction (MI) causes wall thinning and ventricular dilatation, which diminishes the ability of the heart to pump blood, leading to heart failure. This study aims to evaluate the role of placenta-derived mesenchymal stromal cells (pMSC) in the treatment of cardiac failure, as well as its survival after xenogenic transplantation in immunocompetent mice. pMSC were obtained from the chorionic plate of human term placenta, after enzymatic digestion. They were characterized as plastic-adherent and multipotent cells. Flow citometry analysis revealed they did not express hematopoietic (CD45, CD34, CD14, and HLA-DR) or endothelial cells markers (CD31 and CD133), but expressed bone marrow derived MSC-associated molecules (CD90, CD105, and CD73). pMSC also expressed pluripotency markers OCT-4, DMNT3b and KLF4, usually associated to embryonic stem cells, suggesting that their plasticity is superior to that of adult stem cells. Female C57Bl/6 mice were submitted to MI through permanent occlusion of the anterior descending coronary artery. Two weeks

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after MI, mice were submitted to weekly intramyocardial injections, guided through echocardiography, of 105 pMSC or vehicle during 3 weeks. Functional parameters were evaluated weekly by echocardiogram for 40 days after the beginning of treatment. We did not observe significant improvement in ejection fraction, end systolic volume and end diastolic volume in the pMSC group, when compared to the vehicle group. pMSC stably transduced with a viral construct expressing the luciferase gene under the control of MSCV promoter were used in a bioluminescence assay. Cells were detected only at the injection site, suggesting that the injection method is accurate. p-MSC remained at injection site for 3 days after de first injection, but the survival period was reduced after the 2nd and 3rd injection, suggesting immune rejection. Our study indicates that the p-MSCs transplantation does not contribute to improvement in cardiac performance, probably due to the short survival of the cells in the xenogenic setting.

Poster Board Number: 2422

EFFECTS OF DMSO ON GROWTH HORMONE, DIFFERENTIATION AND PLURIPOTENCY MARKER EXPRESSION IN HUMAN STEM CELL CULTURES

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Development process of advanced therapy medicinal products for clinical trials can include a variety of approaches to modulate or increase therapeutical potential of cells. Stem cell modification with small molecular compounds can substantially change expression pattern of various genes, thus potentially influence cell regenerative potential and clinical applicability. The aim of this study was to characterize adult human skin tissue and bone marrow derived mesenchymal stem cell (MSC) populations from different donors and H9 (WA09) hESC culture and to test influences of clinical grade cryoprotectant DMSO on the expression levels of several cytokines, growth factors, their receptors and differentiation/ pluripotency markers. Experiments were carried out on cells cultured in the presence of 1-3% DMSO diluted in media for 48 hours. Dermal and bone marrow derived MSCs were characterized for surface marker expression pattern (positive for CD73, CD90 and CD105; negative for CD45, CD14 and HLA-DR) and differentiation capacity. hESC (H9) were evaluated for the expression of pluripotency markers, cells were passed to the Matrigel coated plates before experiments. The following experimental methods were applied: real time PCR, cytochemistry, ELISA, FACS and HPLC. HPLC analysis demonstrated that DMSO remained chemically stable under cell culture conditions throughout the experiment. We also detected that DMSO had no influence on TERT gene expression and did not affect MSC surface marker expression pattern. DMSO did affect cell differentiation marker pattern, real time PCR showed significantly increased expression for two osteogenic and adipogenic differentiation markers and decreased hondrogenic marker. Flow cytometry analysis showed that presence of 3% DMSO in growth medium of hESC caused significant decrease of pluripotency markers Oct3/4 and Nanog but had minimal effect on SSEA-4. Cytokine panel (IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12, IFNγ and TNFα) expression was tested by PCR. Only IL-6 and IL-12 expression was detected in dermal MSC and DMSO treatment had no effect on this pattern. Dermal MSCs expressed growth factors SDF-1, FGF-2, VEGF and receptors FGF-R, VEGF-R but did not express CXC-R4. Presence of DMSO increased expression of FGF-2 and FGF-R over 4-fold, VEGF increased more than 10-fold, but expression of VEGF-R and SDF-1 was decreased, reaching 4-fold reduction for SDF-1. Expression of the same growth factors was observed in bone marrow derived MSC cultures, which expressed also CXC-R4, and DMSO had similar influence on the expression pattern, except for VEGF that, in contrast, had decrease of expression (4-fold). ELISA assays confirmed corresponding profiles for secreted SDF-1 and VEGF in both tissue MSC cultures. This concept study indicates that high purity DMSO can exert profound effect on expression of several factors which may affect cell culture differentiation and regenerative potential. Therefore use of DMSO and other common and characterized small molecular compounds can be considered as useful approach for design of pre-clinical investigations and clinical trials of adult somatic cells.

Poster Board Number: 2424

X-FACTOR DETERMINES THE INDIVIDUAL VARIATIONS IN THE THERAPEUTIC EFFICACY OF HUMAN UMBILICAL CORD BLOOD DERIVED MESENCHYMAL STEM CELLS

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Objectives- This study was designated to investigate the mechanism underlying the variations in hUCB-MSCs and their impact on therapeutic efficacy in MI model. Background- There is paucity of information whether human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) from separate donors might have different effects on improving myocardial repair after myocardial infarction (MI). Moreover, the underlying mechanism remains elusive. Method and Results- In this study, we established and characterized 4 hUCB-MSCs from 4 different donors. All four umbilical cord blood -derived cells expressed typical human mesenchymal stem cells (hMSC) specific immunophenotypes and immunosuppressive ability in the mixed lymphocyte reaction test. Furthermore, they showed similar morphology, proliferation and differentiation potency. However, hUCB-MSCs showed remarkable variations in the therapeutic efficacy repairing the infarcted rat myocardium 8 weeks after transplantation. To find the signature of the best hUCB-MSC in repairing the infarcted myocardium, we screened cell surface genes at RNA and protein levels. X-factor was the only cell surface gene which was highly expressed in the most efficient hUCB-MSC at RNA and protein levels. Furthermore, X-factor increased VEGF expression via ERK activation in hUCB-MSCs. The infarcted rat heart transplanted with hUCB-MSCs showed human VEGF-positive hUCB-MSCs, human VEGF released from hUCB-MSCs bound to rat endothelial cells, and the enhanced capillary density in peri-infarct area. Conclusions- Human hUCB-MSCs showed a remarkable individual variation in repairing infarcted myocardium. X-factor could be used as a signature molecule in selecting hUCB-MSCs having a good therapeutic efficacy.

Poster Board Number: 2426

IMMUNOLOGICAL PROPERTIES HUMAN ADIPOSE-DERIVED MESENCHYMAL STROMAL CELLS UNDIFFERENTIATED AND PREVIOUSLY COMMITTED TO MYOGENIC PHENOTYPE

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Progressive muscular dystrophies (PMD) are a group of disorders characterized by progressive degeneration of skeletal muscle caused by the absence or defective muscular proteins. The possibility to use cell therapy for the treatment of PMD has been investigated by many groups. We have recently shown that undifferentiated human adipose-derived stem cells (hASCs) can

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differentiate into skeletal muscle when in contact with dystrophic muscle *in vitro* and *in vivo*; these cells were not rejected after systemic injection even without immunosuppression. However, surprisingly when these cells were injected systemically after being cultured in myogenic induced media no human cells were found in the host muscles. In order to better understand this observation we compared the effect of undifferentiated and myogenic committed hASCs on the proliferative function of mouse lymphocytes as a measure of the immune response (mixed lymphocyte culture). We also evaluated if the systemic injection of undifferentiated and myogenic committed hASCs modifies the function of the mouse lymphocytes. Our results confirm that undifferentiated hASCs are immune modulating cells but myogenic committed hASCs lose these properties and cause lymphocyte growth. These observations suggest that allogenic and xenotransplanted hASCs will not cause an immune response as long as they are undifferentiated which may have important applications for future therapy in patients with different forms of PMD. Supported by FAPESP-CEPID, INCT and CNPq.

Poster Board Number: 2428

FUNCTIONAL PROPERTIES OF BONE MARROW MESENCHYMAL STEM CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS

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Mesenchymal stem cells (MSC), usually extracted from bone marrow, play several physiological roles, including angiogenesis induction. Taking into consideration the defective angiogenesis and microvascular rarefaction in systemic arterial hypertension (SAH), it could be hypothesized that MSCs could participate in the pathogenesis of SAH. The aim of the present study was to evaluate some functional aspects of bone marrow-derived MSCs in spontaneously hypertensive rats (SHR) comparing with normotensive control Wistar-Kyoto (WKY) rats. After confirmation of arterial blood pressure levels in age-matched (16-24 weeks) SHR and WKY rats, MSCs were extracted, cultured, phenotypically characterized by flow cytometry and submitted to some functional assays: a) colony-forming unit-fibroblastic (CFU-F) assay, b) proliferation assay, c) doubling populations *in vitro* assay and d) differentiation assay in osteogenic and adipogenic lineages. Number of CFU-F from SHR was similar to that from WKY. MSCs proliferation analysis shows a great rise in cells number with the time, which was significantly lower in SHR, indicating a proliferative dysfunction of MSCs in SHR. According to doubling-population *in vitro* assay, MSC from SHR were doubled 27 times in culture, while MSCs from WKY were doubled 42 times until acquire senescent phenotype. At the end, *in vitro* differentiation assay revealed a lower ability of MSCs from SHR to differentiate in osteogenic and adipogenic lineages. The results showed a functional deficit of bone marrow-derived MSCs from SHR. The possible mechanisms and eventual consequences of this functional deficit for the hypertensive state are not known and deserve intensive future investigation.

Poster Board Number: 2430

BONE MARROW-DERIVED MESENCHYMAL STEM CELLS ATTENUATE PROGRESSION OF LATE STAGE CHRONIC KIDNEY DISEASE IN RATS

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In recent years, cell therapy with bone marrow-derived mesenchymal stem cells (BM-MSC) has been intensively investigated in a pre-clinical context using animal models of chronic kidney disease (CKD). However, BM-MSC administrations are performed in the first days or weeks after lesion in these animal models, which are not a good surrogate of real clinical conditions. Therefore, our aim was to investigate the effects of BM-MSC administration in remnant kidney model in a late-stage of the disease. Forty eight female 12-16 weeks old Wistar Kyoto rats were divided in three groups: two of them were submitted to 5/6 nephrectomy to induce CDK (groups NX-VE, receiving vehicle and NX-MSC, receiving BM-MSC) and one group were submitted to a sham surgery (SHAM-VE). BM-MSC were extracted from femur and tibiae from donor male age-matched Wistar Kyoto rats (n=10) and cultivated *in vitro* on appropriated conditions. All animals were weekly followed taking measures of systolic arterial pressure and heart rate by means of tail occlusion method. After 8 weeks, plasma and 24 hours urine samples were collected to measure creatinine clearance and proteinuria, and then animals were transplanted with 5 x 106 MSCs (NX-MSCs, n=8) or vehicle (NX-VE, n=12), while sham animals received vehicle (SHAM-VE, n=12). Five weeks after transplantation, all animal were submitted to baseline arterial pressure recordings followed by arterial baroreflex test and pharmacological blockade with atropine and propranolol to calculate sympathetic and parasympathetic tonus. After 24 hours, all animals, under anesthesia, were submitted to an endothelial function study measuring endothelium-dependent and endothelium independent vasodepressor responses. At the ending, animals were sacrificed and organs like remnant kidney, heart, lungs, aorta, spleen and skeletal muscle were collected and stored in -80oC freezer. Morphological and morphometrical studies of remnant kidney were performed to measure nephron numbers, glomerular diameter, inflammatory infiltrate, glomerusclerosis and interstitial fibrosis. Our results demonstrated that BM-MSCs expressed positive markers for CD29 and were negatives for CD11b, CD34, CD31, CD45 e c-kit. In addition, these cells are able to differentiate in adipogenic and osteogenic lineages. During all study period, body weights were not different among groups while systolic pressure of NX-VE animals was higher than SHAM-VE animals and treatment with BM-MSC reduced systolic pressure in NX-MSC animals. These animals also presented improvement in renal function measured as reduction in plasma levels of urea and creatinine and proteinuria, as well as increase in creatinine clearance. Baroreflex sensitivity and autonomic tonus did not differ among groups. Endothelium-dependent vasodilation mediated by acetylcholine was significantly improved in NX-MSC when compared to NX-VE animals. Regarding to remnant kidney morphology, BM-MSC played a protective role preventing nephron loss and hypertrophy, as well as blunting inflammatory infiltrate and glomerular and interstitial fibrosis. In conclusion, BM-MSC transplantation, even in a later stage of CKD, was able to efficiently improve or delay progressive renal damage, possibly via their paracrine and immunomodulatory effects, promoting significant improvements in renal function and structure. This cell therapy with BM-MSC could represent an efficient alternative for the treatment of CKD in a near future.

Poster Board Number: 2432

MYOGENIC POTENTIAL OF MURINE MESENCHYMAL STEM CELLS FOR THERAPY IN PROGRESSIVE MUSCULAR DYSTROPHIES

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Human muscular dystrophies (MD) are a heterogeneous group of genetic disorders, caused by mutations in genes coding for sarcolemmal, sarcomeric and citosolic muscle proteins. Deficiencies or loss of function of any of these proteins lead to irreversible and progressive degeneration of skeletal muscles, resulting in a variable degree of weakness. Since no effective treatment is available for MD, mouse models for these diseases are valuable tools for testing putative cell therapies. Murine bone-marrow mesenchymal

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stem cells (BM-MSC) and adipose tissue mesenchymal stem cells (ASC) are multipotent cell populations that show immunosuppressive properties, an important characteristic to be considered in their use for transplantation. The main objective of this study was to evaluate and compare the myogenic potential of mesenchymal stem cells from these two different sources *in vivo*, in three different mouse models for dystrophy: Dmdmdx (total absence of dystrophin, model for Duchenne MD), Lama2dy2J/J (α2-laminin deficient, severe phenotype, model for congenital MD type 1A) and Largemyd (defect in glycosylation of α-DG, severe phenotype in older animals, model for congenital MD type 1D). Aiming to study homing and behavior of these cells under non-dystrophic conditions, normal C57Black mice were also injected. BM-MSC and ASC were isolated from eGFP-FVB/N mice and characterized by flow cytometry for stem cell markers before injections. 36 animals were injected weekly for one month through systemic intravenous delivery as followed: 22 animals injected with BM-MSC - 3 C57Black, 7 Dmdmdx, 6 Largemyd and 6 Lama2dy2J/J and 14 mice injected with ASC - 4 C57Bl, 4 Dmdmdx, 3 Largemyd, and 3 Lama2dy2j/J. The presence of donor cells at the host muscle was evaluated by PCR for the DNA of the eGFP construction and dystrophin protein expression was verified by western blotting. We found DNA of transplanted cells in 10 out of the 22 injected animals with BM-MSC (1 C57Bl, 4 Dmdmdx, 1 Largemyd and 4 Lama2dy2j/J) and in 8 of 14 mice treated with ASC (2 C57Bl, 3 Dmdmdx, 1 Largemyd and 2 Lama2dy2j/J). These results show a proper engraftment of the normal cells in the target tissue both for hostile dystrophic environment as well as for the normal muscle after systemic delivery. Additionally, the injected cells were retained despite the known differences in MHC H2 haplotype of donor and receiver mice. Comparing the two sources, ASC showed a slightly better proportion of maintenance in comparison with BM-MSC (57% of retention versus 45%). However, both sources could be considered for cell therapy targeting muscular dystrophies, regarding cell retention. At the protein level, we could detect a small amount of dystrophin expression in the eGFP positive muscles from Dmdmdx mice, mainly the ones injected with ASC, suggesting thus that this source possibly presents a better myogenic potential. Complementary additional studies, which are currently underway, are necessary to improve the amount of expressed muscle proteins leading to an enhanced therapeutic effect. FAPESP-CEPID, CNPq-INCT, FINEP, ABDIM.

Poster Board Number: 2434

ROLES OF MATRIX METALLOPROTEINASE AND MESENCHYMAL STEM CELL THERAPY IN ISCHEMIC STROKE

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In the ischemic stroke, beneficial mechanism of mesenchymal stem cells (MSC) is largely unknown. Neurogenesis, angiogenesis, neuroprotection, and activation of endogenous neurorestorative processes had been suggested. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases and they can destruct extracellular matrix. Detrimental effects of MMPs are well known after ischemic stroke. MMPs is strongly associated with the hemorrhagic transformation, edema formation and thrombolytic failure in the ischemic stroke. Recent reports showed that MMPs have a role in the recovery phase after stroke. Moreover, bone marrow-derived cell is a major supplier of MMP and crucial for vascular endothelial growth factor (VEGF) induced angiogenesis. This study sought the roles of MMPs in MSC therapy after ischemic stroke and the best time of MSC injection. The experiments were conducted through two phases. In phase 1, we searched the appropriate injection time of human MSC (hMSC) after middle cerebral artery occlusion (MCAO). The injection was performed to male Sprague-Dawley rats at three different time points (1 hr, 1 day, or 3 days). In phase 2, the serial changes of neurological improvements, activities of MMPs and infarction volumes after hMSC or vehicle injections were investigated. In phase 1, hMSC injection at 1 hr after MCAO group showed marked neurological improvement in the rotarod tests ($p = 0.023$) and Longa score ($p = 0.018$). The activities of MMP-2 was increased in the 1 hr hMSC injection group compared with control (3130 ± 1807 vs. 1087 ± 579, $p = 0.028$), whereas MMP-9 levels were not different between hMSC injection and control groups. In phase 2 experiments, the group of hMSC injection at 1 hr after MCAO showed significant neurological improvement on rotarod test ($p = 0.027$) and Neurological Severity Score ($p = 0.001$) compared with vehicle injection rats. In gelatin zymography, the MMP-2 activities at 1 day were higher in the hMSC groups than the control (828 ± 119 vs. 360 ± 203, $p = 0.002$). No difference of MMP-9 activity was detected. The Infarctions volumes were not different between the hMSC-treated rats and control ($p = 0.625$). In this study, a favorable neurological outcome after the hMSC injection was shown, particularly after earlier injection of hMSC. Higher MMP-2 level was noted in the 1 hr hMSC injection after MCAO. Neurological improvements without reduction of infarction volumes may suggest that the beneficial effects of hMSC are related from the increased plasticity. The brain plasticity may be associated with earlier MMP-2 elevation after hMSC injection in the ischemic stroke.

Poster Board Number: 2436

EVALUATION OF GENE TRANSFERRING EFFICIENCY VIA PAMAM G6 DENDRIMER AS A GENE PORTER TO MESENCHYMAL AND EMBRYONIC STEM CELLS

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Gene transfer to stem cells is still one of the challenging issues in the field. Although, the best transduction efficiency is resulted from viral gene transduction, but the remnant viral genome can impair the physiology of infected cells and in some cases it may cause mutagenesis. In the other hand, for efficient gene transfer via electroporation, the cells must be dissociated by enzymes which can irreversibly damage the aggregation and in turn survival of human embryonic and induced pluripotent stem cells. Given the mentioned obstacles, non-viral gene transfer by chemical vehicle, may still attend as an alternative approach for gene transferring to stem cells. In present study, the efficiency of polyamidoamine (PAMAM) G6 dendrimer, as a chemical gene porter, was assessed in gene transferring to mesenchymal (MSC) and embryonic stem cells (ES) and the efficiency was compared to lipofectamine. To achieve this goal, a GFP-containing plasmid was trandfected to stem cells using PAMAM G6 denderimer or lipofectamine. Three days after, the number of GFP positive stem cells was quantified by flow cytometry. The result showed PAMAM G6 has more transfection efficiency than lipofectamine in gene transferring to MSC, whereas in ES cells, the lipofectamine showed better transfection results. Cytotoxicity studies by MTT revealed that PAMAM G6 dendrimer has lower cytotoxicity than lipofectamine in transfected MSC but in contrary, it showed a higher cytotoxicity in ES cells. Taking into account the above results with the cheap cost of PAMAM G6 dendrimer, our study pointed PAMAM G6 as a promisimg carrier for gene transferring into mesenchymal and embryonic stem cells.

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Poster Board Number: 2438

MURINE MESENCHYMAL STEM CELLS HAVE A DISTINCT PROTEIN ISOFORM SIGNATURE FROM THAT OF NON-MULTIPOTENT STROMA.

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Cell function is often controlled by dynamic changes in the expression of different protein isoforms. In the current study, we utilized C57Bl/6 mouse bone marrow stromal cultures, together with quantitative mass spectrometry (MS/MS), to identify protein isoforms that may play a role in regulating the proliferation, differentiation and immune suppressive functions of mesenchymal stem cells (MSC). Proteins isolated from the nucleus and cytoplasm of stem cell enriched (CD105+) and depleted (CD105-) stromal cells were labelled with either deuterated or non-deuterated formaldehyde and analyzed by liquid chromatography MS/MS. Quantitative comparison of 123 protein isoforms was completed between the two cell populations. Thirty-four isoforms were identified that were expressed at two-fold greater or lower levels in MSC. Where possible, proteomic analysis was verified by western blotting and was demonstrated to be divergent from the level of gene transcription detected for certain proteins. Our analysis provides a protein isoform signature specific to MSC and a basis for understanding the protein pathways involved in the regulation of these therapeutically relevant stem cells.

Poster Board Number: 2440

SSEA-4 IS NOT A MARKER OF MESENCHYMAL STEM CELLS IN CULTURE.

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Bone marrow derived stromal cultures contain multipotent mesenchymal stem cells (MSC) that can initiate tissue repair and suppress the immune system. Consequently, the therapeutic efficacy of stromal cultures are currently being tested in clinical trials for a wide range of diseases and tissue damage. The identification of markers that discriminate MSC from non-multipotent stroma would provide a means for determining the efficacy of these cultures prior to therapeutic use. The goal of the present study was to determine whether SSEA-4, a known marker of MSC *in vivo*, is expressed on multipotent stromal cells *in vitro*. Standard and serum free stromal cultures were created from the bone marrow of 4 separate donors and characterized for SSEA-4 expression and multipotent differentiation potential. The proportion of SSEA-4 expressing (SSEA-4pos) cells varied from 9% to 89% in both the presence and absence of serum. Purified SSEA-4pos and SSEA-4 non-expressing (SSEA-4neg) cells were each capable of differentiating into fat, bone and cartilage and showed no difference in proliferating potential over a 60 day culture period. After 7-10 days, cultures derived from either purified population consisted of a mixture of both SSEA-4pos and SSEA-4neg cells at proportions similar to that found in the original culture. To provide a quantitative comparison of MSC, clonal cultures are currently being derived from SSEA-4pos and SSEA-4neg cells and characterized in multipotent differentiation assays. Our data demonstrate a divergence between the SSEA-4 phenotype of MSC *in vivo* compared to *in vitro*, suggesting that SSEA-4 expression will not provide a suitable marker for measuring the medicinal component in therapeutically relevant stromal cultures.

Poster Board Number: 2442

STROMAL CELLS IN AMNIOTIC FLUID: EVIDENCE FOR DERIVATION BY EPITHELIAL TO MESENCHYME TRANSITION

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Neural damage due to trauma, disease and aging limits the quality of life for millions worldwide. Mesenchymal stromal cells (MSCs) have garnered significant interest in regenerative medicine as a source of trophic support for self-repair and immunomodulation of the damaging effects of inflammation. Long lived stromal cells in amniotic fluid (AF) resemble MSCs derived from bone marrow (BMMSC); both cell types differentiate into connective tissues such as fat, bone, and cartilage and express a broad spectrum of trophic factors, cytokines, and immunomodulatory proteins. Neither the source of cells in AF nor the precise identity of the AF cell types responsible for BMMSC-like activities is well established. Motivation for the current study was to determine 1) the source of stromal cells in AF and 2) to determine whether trophic activity is limited to specific clonal lineages. We first generated clonal lines by simple dilution (1:10 - 1:500) of 4 uncultured amniocentesis samples in multiwall plates; 43 independent clonal lines expanded from a single point source. Clonal populations were classified by phase microscopy as either stromal-like or epithelial-like. Selected epithelial-like clones failed to proliferate beyond 10-12 passages. While the proliferative capacity of stromal cell populations declined over time, representative lines could be maintained for 15 to 30 passages before senescence. Total RNA was isolated from representative clones of stromal cells, epithelial cells and the previously established A1AFS cell line of MSC-like cells. A preselected set of genes encoding growth factors, cytokines and immunomodulatory proteins were assayed by ABI microfluidics gene arrays and validated by individual ABI TaqMan assays. Transcript profiles showed differences between lines, but all lines tested expressed a broadly overlapping range of factors that could promote self-repair. We developed a neural cell-based assay to correlate transcript profiles with cell-based trophic activity; proliferating cultures of neural progenitors were generated from our ChM5 iPS cells, a fully validated and vector-free line of induced pluripotent stem cells. In the absence of extrinsic neurotrophic factors and mitogens, neural progenitors cease proliferation and differentiate into neurons. Media conditioned by stromal cell clones, but not epithelial cell clones, improved viability of neurons in differentiating cultures of neural progenitors in comparison to comparably aged control media, suggesting that trophic support may be an activity of stromal cells. Given that MSCs can be generated by epithelial to mesenchmal transitions, this same set of stromal cell clones and BMMSCs were evaluated by iimmunostaining for markers of stromal and epithelial cell types. Immunostaining showed profiles typical of MSCs, but a subset of stromal cell clones also showed epithelial cell characteristics, including extensive networks of keratin as well vimentin and E-cadherin. Together with other studies, these findings raise the question of whether AF-derived populations can reflect epithelial to mesenchyme transitions of cells released from epithelial exposed to amniotic fluid. Given the results of our neural cell based assays, our results further suggest that the source of stromal cells may not be critical for trophic activity.

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MESENCHYMAL STEM CELL DIFFERENTIATION

Poster Board Number: 2444

HUMAN INDUCED PLURIPOTENT STEM CELLS DIFFERENTIATE INTO OSTEO- AND CHONDROGENIC PROGENITORS

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Multipotent human mesenchymal stem cells (MSCs) have been identified as promising candidates for advancing drug discovery and cellular therapy strategies, especially for the correction of disorders of orthopedic origins. Characterized by fibroblast-like morphology and isolated from multiple sources like adult bone marrow, adipose tissue and umbilical cord blood, human MSCs are typically distinguished by their ability to differentiate along multiple pathways including osteogenic, chondrogenic and adipogenic lineages. Despite their broad potential, MSCs pose significant problems for the researcher, including: 1) surgical procedure required for initial patient harvest; 2) variability in starting population; 3) limited lifespan *in vitro*; 4) loss of multilineage capacity soon after continued propagation; and 5) heterogeneous multilineage differentiation potential depending on tissue and donor source. Hence, developing homogeneous cultures of mesenchymal-like cells derived from induced pluripotent stem cells (iPSCs) will circumvent the drawbacks of adult MSCs and offer a theoretically limitless supply of genetically identical and patient-specific stem cells. Derived from reprogrammed adult somatic cells, iPSCs are indistinguishable in their epigenetic state and developmental potential from blastocyst-derived embryonic stem cells (ESCs). However, the capacity of iPSCs to differentiate into therapeutically relevant and functional cell types in comparison to ESC-derived cell types and adult stem cell sources is largely uninvestigated. Here we generated human iPSCs by transducing IMR-90 human lung fibroblasts with lentiviral vectors encoding 6 pluripotency factors, OCT3/4, NANOG, SOX2, KLF4, LIN28, and c-MYC. In these studies we induced iPSCs to differentiate *in vitro* into mesenchymal-like cells and derivative phenotypes using ArunA's unique culture system. Resulting progenitor cells were characterized by flow cytometry, real-time PCR and then assayed for differentiation potential in comparison to hESC-derived progenitors and bone marrow and umbilical cord sources of MSCs. We found that the morphology, time frame for differentiation and marker expression of iPSC-derived progenitors is similar to hESC-derived progenitors for mesenchymal differentiation *in vitro*. Interestingly, we also found that iPSC-derived progenitors mimic umbilical cord MSCs in their lineage potential, showing a greater capacity for osteogenic differentiation in contrast to bone marrow MSCs with higher adipogenic capabilities. Our results show ArunA's culture system enables the large-scale production of an osteo/chondrogenic lineage-restricted cell line from iPSCs, capable of being maintained as robust, adherent monolayer cultures for multiple passages, with distinctive utility for future orthopedic regenerative medicine applications.

Poster Board Number: 2446

BORTEZOMIB PROMOTES OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

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Human mesenchymal stem cells (hMSCs) are multipotent cells which possess the osteogenic differentiation potential that could be used for bone and cartilage reconstruction. During the past few years, drug targeting of adult stem cells has been proposed as a strategy for regenerative medicine, but the use of such strategy to augment the osteogenic differentiation of hMSCs is still poorly explored. It has evidence that the proteasome inhibitor Bortezomib (BZB) (a chemotherapeutic drug which is approved for treating patients with multiple myeloma) could induce the differentiation of mouse MSCs toward osteocyte lineages. In this study, we have studied the effects of BZB on the osteogenic differentiation process of hMSCs. The isolated hMSCs from bone marrow expressed typical MSC markers, being positive for CD105, CD90 and CD73, and negative for CD45 and CD34 and were able to differentiate to osteocytes and adipocytes *in vitro*. The results showed that 1-2 nM BZB promoted osteogenic differentiation of hMSCs as indicated by the level increasing of alkaline phosphatase (ALP) activity and the up-regulation of early osteogenic marker genes, such as runt DNA-binding domain transcription factor-2 (RUNX2) compared with controls. Moreover, after 28 days, BZB could trigger the matrix mineralization processes of hMSC-derived osteocytes (a crucial step towards the formation of calcified extracellular matrix associated with functional bone) as evidence by the positive increasing of Alizarin Red S staining and the up-regulation of osteocalcin (OCN) gene in comparison to controls. In summary, these results suggest that BZB, an anti-cancer drug, could induce the osteogenic differentiation of hMSCs and might have a potential to be used in several clinical applications, such as tissue engineering and cell-based therapy in the near future.

Poster Board Number: 2448

POLYAMINES PROMOTE OSTEOGENIC DIFFFERENTIATION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Polyamines are organic polycations that are derived from amino acids. These molecules of low molecular weight are ubiquitous in all organisms, and are essential for cell proliferation and differentiation. Eukaryotes contain the three common polyamines, putrescine, spermidine, and spermine, while in bacteria, the principal polyamines are putrescine, cadaverine and spermidine, but a large range of uncommon polyamines also exists. Several early studies have implicated the relationship of polyamines to the growth and development of bone and cartilage. More recently, spermine was shown to promote osteogenic differentiation of goat mesenchymal stem cells. Nevertheless, the mechanisms underlying the osteogenic potential of polyamines remain

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unclear. In this study, we found that all three of the common eukaryotic polyamines, putrescine, spermidine, and spermine, were capable of promoting osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs). Extracellular matrix mineralization, a marker for osteoblast maturation, was enhanced in the presence of polyamines. Besides, alkaline phosphatase activity and the expression of osteogenic genes, such as BMP2, Runx2, osteopontin and osteocalcin, were up-regulated, suggesting that polyamines may promote osteoblastogenesis of hBMSCs through BMP2- and Runx2-related pathways. Developing new drugs to enhance osteogenesis is crucial in orthopedic research. Currently, the only drug used clinically to stimulate bone formation is parathyroid hormone (PTH), which possesses the risk of inducing osteosarcoma. Studies on polyamines as novel osteogenic inducers not only help to explore the mechanism of their osteogenic activity and their efficacy in the treatment of skeletal disorders such as osteoporosis, but also accelerate the development of polyamine-derived new drugs that stimulate bone formation.

Poster Board Number: 2450

OSTEOBLAST DIFFERENTIATION OF HUMAN AMNIOTIC FLUID-DERIVED STEM CELLS IRRADIATED WITH VISIBLE LIGHT

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Cells have been recognized to be sensitive to visible light, although most of research introduced light-activated channels or enzymes into the cells genetically for the investigation of effect of visible light on the cell function. There are also several studies that investigated the effect of light on the regulation of cell functions of native cells (e.g., genetically unchanged cells). However, there are only few studies to investigate the effect of light irradiation on stem cells. Here we repot effect of light irradiation of visible light on the expression of pluripotent genes (Oxt-4, Sox2, and Nanog) in human amniotic fluid-derived stem cells (hAFSCs) and on the osteogenic differentiation ability of hAFSCs under light irradiation from light emitting diode (LED) having several wavelength of light, blue (470 nm), green (525 nm), yellow (600 nm), and red (630 nm) light at 0-2 mW/cm2. Pluripotent gene expression in hAFSCs was upregurated by the irradiation of visible light (blue, green, and yellow light) from LED more than 6 hours, while no enhanced expression of pluripotent genes in AFSCs under irradiation of red light was observed. Green light irradiation to hAFSCs upregulated the pluripotent genes significantly than irradiation of blue, yellow and red light. Light irradiation of specific wavenumber is found to be important to upregulate the pluripotent genes in hAFSCs.The osteogenic differentiation of hAFSCs was evaluated in differentiation medium of osteoblasts under irradiation of LED light for 0 or 48 hr to investigate the effect of light irradiation on the osteogenic differentiation of hAFSCs from alizarin red S staining (calcium deposition), alkali phosphatase activity (early osteoblast marker), and gene expression of osteopontin (late osteoblast marker). It was found that alkali phosphatase activity and alizarin red S staining area as well as the gene expression of osteoblast differentiation marker in hAFSCs increased when blue light was irradiated on AFSCs at light intensity more than 1 mW/cm2. Therefore, the intensity of light irradiation on AFSCs was fixed to be 1 mW/cm2 in the following experiments. The osteogenic differentiation of hAFSCs was significantly facilitated by green and blue light irradiation from LED, but not significantly by red and yellow light. Light irradiation of specific wavenumber is found to be important to facilitate the osteogenic differentiation of hAFSCs. One possible mechanism for the facilitated differentiation of hAFSCs into osteoblasts by irradiation of visible light is mediated by reactive oxygen species (ROS), which have been shown to be secondary messengers in cell signal transduction. Therefore, we have evaluated the differentiation ability of hAFSCs into osteoblasts irradiated by blue light (24hr, 1 mW/cm2) under existence of free radical scavenger in the differentiation mediumin. The facilitated differentiation into osteoblasts by irradiation of visible light was found not to be mediated by ROS, because alkaliphosphatase activity and gene expression of osteopontin did not change significantly among hAFSCs in the differentiation medium with and without scavenger (vitamin C). The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK), MAPK/ERK pathway as well as other unknown signal pathways should be responsible for the activation of signal pathways for the facilitated differentiation of hAFSCs into osteoblasts on light irradiation.

Poster Board Number: 2452

HUMAN AMNIOTIC FLUID STEM CELLS CAN FUNCTIONALLY DIFFERENTIATE ALONG SMOOTH MUSCLE LINEAGE

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Introduction - In order to create a biocompatible tissue to replace absent or damaged organ, the generation of a functional smooth muscle (SM) layer is essential. In our study we aimed to derive functional SM cells from human Amniotic Fluid Stem (hAFSC) cells. Materials and Method - Clonal lines were generated by limiting dilution from c-kit+ hAFSC. Differentiation towards SM lineage (SMhAFSC) was obtained using a medium conditioned by PDGF-BB and TGF-b1 addition. hAFSC were transduced using vectors encoding both ZsGreen and DsRed respectively under the aSMA and MHC promoter. qPCR was used to evaluate aSMA, desmin and calponin and smoothelin expression. Immunofluorescence was performed for a-SMA, desmin, smoothelin, CD31 and pancytokeratine. To better understand ultrastructural features, Transmission Electron Microscopy (TEM) analysis was also carried out. Cells were analysed by cytofluorimetry and time-lapse analyses under carbachol/atropine were performed to evaluate single cell contractility. Ultimately calcium activated potassium channels were assessed by patch-clamping and functionality was validated by collagen lattice assay (CLA). Results - SMhAFSC expressed significantly higher level of aSMA, desmin and calponin transcripts after selective culture condition. These features were confirmed by immunofluorescence, demonstrating a single lineage commitment. TEM confirmed increased intermediate filaments, dense bodies and glycogen deposits in SMhAFSC. Sequential imaging analyses demonstrated that SMhAFSC have a higher contractile potential than hAFSC. Consecutive single cell sampling showed the presence of voltage-dependent calcium activated potassium channels on differentiated SMhAFSC, which are sensitive to tetraethilammonium blockade, inhibited by angiotensin II and activated by carbachol (as in functional SM cells). By contrast undifferentiated hAFSC did not respond to mediators. Lastly SMhAFSC confirmed an enhanced contractility by CLA. Conclusion - hAFSC under selective cultural conditions are able to give rise to functional SM cells and transduction represents a valuable tool to select SM committed population. This step may eventually overcome the well-known problem of expanding SM progenitors, making these cells amenable to tissue engineering.

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Poster Board Number: 2454

COMPARATIVE CHARACTERIZATION OF MULTIPOTENT CELLS ISOLATED FROM HUMAN AMNIOTIC MEMBRANE AND PLACENTA

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Fetal tissues such as placenta, cord, and amnion are temporary tissues in supporting fetus development during pregnancy. Recently, we isolated the multipotent stem cells from human term placenta (hPDMCS) and showed these progenitors were with plasticity in differentiating toward multiple lineage cells. We were wondering whether other fetal tissues such as amniotic membrane contain multipotent progenitors. Trypsin-EDTA disassociated amnion cells were cultivated and isolated through the abilities in adhering to plastic surface. Some colony-forming fibroblastic cells were observed s on the bottom of culture vessels. Our results demonstrated these cells were with the differentiation abilities even after 30 passages. In this study, we compared the properties and differentiation potentials of placenta- and amniotic membrane-derived progenitors. Both progenitors were with fibroblast-like morphologies and positively expressed CD90, CD105, SH3, SH4, CD29, CD44, STRO-1 and absent for CD45 and CD34. In contrast to placenta-derived progenitors, the immunofluorescent staining results revealed that c-Met could not be detected on amniotic membrane-derived cells. The differentiation assays showed both stem cell populations were capable in differentiating toward adipocytes and osteocytes. However, the hepatic differentiation experiments showed the amniotic membrane isolated progenitors were able to convert to hepatocyte-like cells which could be positively stained by anti-Hep Par1 antibodies. According to our results, progenitors derived from amniotic membrane and placenta shared similarities on their immunophenotypes and possess comparable differentiation capabilities.

Poster Board Number: 2456

MICRORNAS-181A MODULATES HUMAN MESENCHYMAL STEM CELLS DIFFERENTIATION INTO CARDIOMYOCYTE THROUGH TARGETING OF PROTEIN KINASE C

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Understanding the molecular mechanisms that regulate cellular proliferation and differentiation is a central theme of developmental biology. MicroRNAs (miRNAs) are a class of regulatory RNAs of approximately 22 nucleotides that post-transcriptionally regulate gene expression. Increasing evidence points to the potential role of miRNAs in various biological processes. Cell fate is likely to be controlled by an elaborate orchestration of multiple signaling pathways. In a previous study, we reported the development of cardiogenic cells from rat MSCs activated by phorbol myristate acetate (PMA), a PKC activator, which exhibited high expressions of cardiac-specific markers (cTnT, MHC, MLC) and Ca2+ homeostasis-related proteins. Here we confirmed that cardiogenic differentiation led to in PMA-treated human mesenchymal stem cells (hMSCs). We found that miR-181a down-regulates the expression of PKC by targeting sequences in the 3'UTR of PKC mRNA. The overexpression of miR-181a regulates expression of PKC which are involved in cellular processes linked to differentiation. To investigate the role of miR181a in differentiation, hMSCs were transfected with miR181a mimic. cTnT expression level is not exchange in miR-181a transfected cell, indicates that the ectopic expression of miR-133a maintains MSC properties, whereas the miR-181a inhibitor increase cTnT, MHC and MLC expression. We conclude that miR-181a has major roles in cardiac differentiation though direct targeting of PKCs genes.

Poster Board Number: 2458

MICRORNA-23B INDUCED CHONDROGENIC DIFFERENTIATION FROM HUMAN MESENCHYMAL STEM CELL BY MODULATING PROTEIN KINASE A

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MicroRNAs (miRNAs) are short non-coding RNA molecules that fulfill a variety of biological functions by negatively regulating gene expression. Critical regulation of protein expression is important for controlling the balance between self-renewal and differentiation in stem cells and cellular processes are regulated by an elaborate orchestration of multiple signaling pathways. H-89 (PKA inhibitor) inhibit cAMP/Protein kinase A (PKA) pathway leading to suppress phosphorylation of cAMP response element binding (CREB) family of transcription factors. We have shown previously that H-89 induced the chondrogenic differentiation of rat mesenchymal stem cells (MSCs). Here, we confirmed the involvement of H-89 in the chondrogenic differentiation of hMSCs and found that miR-23b induce chondrocyte differentiation through down-regulation of PKA signaling. To determine the relation of miR-23b and PKA in chondrogenic differentiation, we examined that miR-23b expression on H-89-treated hMSCs by real time-PCR and that effect of miR-23b on PKA regulation by western blot analysis. MiR-23b is up-regulated upon chondrogenic differentiation by H-89 and overexpression of this miRNA on hMSCs decreased protein levels of PKA and p-CREB. Reporter gene analysis was confirmed the functionality of the miR-23b target in the 3'-untranslated region of PKA mRNA. Finally, we detected significantly increased chondrogenic phenotype on miR-23b transfected hMSCs using measuring absorbance of Alcian blue and expression of aggrecan, a chondrocyte marker. Also, integrin α5β1, fibronectin, and N-cadherin were down-regulated in miR-133a transfected hMSCs. These results indicate that miR-23b contributes to the regulation of chondrogenic differentiation by negatively inhibition of PKA signaling.

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Poster Board Number: 2460

MICRORNAS 26A IS POTENT INDUCERS OF MESENCHYMAL STEM CELL DIFFERENTIATION INTO ENDOTHELIAL CELLS THROUGH TARGETING OF GLYCOGEN SYNTHASE KINASE 3β

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MicroRNAs function as negative regulators of gene expression by inhibiting the translation or promoting the degradation of target mRNAs, having major roles in cellular differentiation. Cell fate is likely to be controlled by an elaborate orchestration of multiple signaling pathways. We found miR-26a to be highly overexpressed in during differentiation. To investigate the role of this miRNA in differentiation, human mesenchymal stem cell (MSC) were transfected with miR-26a mimic. Overexpression of miR-26a led to the expression of CD31, an endothelial specific marker, which was similar to that induced by SB216763 in human MSCs. Also, SB216763 increased mRNA level of eNOS, VCAM-1, VE-cadherine and VEGF-R2, endothelial specific markers. One of the predicted downregulated miR-26a targets was glycogen synthase kinase 3β (GSK-3β), target of SB216763, which is involved in cellular processes linked to either proliferation or differentiation. GSK-3β was experimentally validated as a direct target of miR-26a, and siRNA-mediated inhibition of this mRNA alone resulted in expression of CD31, VE-cadherine and VEGF-R2. Moreover, we show that miR-26a could be stabilized β-catenin by overexpression of this miRNA. We conclude that miR-26a has major roles in the process of mesenchymal stem cell differentiation through direct targeting of GSK-3β, which induces the downregulation of β-catenin.

Poster Board Number: 2462

MICRORNA-133 PROMOTES CARDIOMYOCYTE DIFFERENTIATION BY MODULATING EPIDERMAL GROWTH FACTOR RECEPTOR

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MicroRNAs (miRNAs) are known to cause translation inhibition or degradation of targeting mRNAs, as important posttranscriptional regulatory roles. miRNAs play essential roles in cell fate through multiple signaling pathway by a delicate orchestration. Among various miRNAs, we found miR-133 to be involved in cardiac differentiation and investigated the roles of miRNA-133 transfecting with miR-133 mimic to human mesenchymal stem cells (MSCs). The overexpression of miR-133 by transfection of mimic increased the mRNA expression level of a cardiac-specific markers (cardiac Toroponin T; cTnT, myosin light chain; MLC, myosin heavy chain; MHC, atrial natriuretic peptides; ANP, brain natriuretic peptide; BNP) similar to expression level of human MSC-treated by compound 56 (4-[(3-Bromophenyl)amino]-6,7-diethoxyquinazoline), a specific inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR). EGFR was one of predicted miR-133 targets as the target of compound 56 which is involved in the regulation of several cellular modifications leading to biological activity. We confirmed the target of miR-133 through luciferase assay and validated that the protein expression of EGFR is mediated by miR-133 mimic. Taken together, we conclude that EGFR is one of miR-133 targets and miR-133 has crucial roles in mediation of human MSC differentiation via targeting of EGFR.

Poster Board Number: 2464

MIR-210 MODULATES HYPOXIA INDUCIBLE FACTOR 1 INDUCED SURVIVAL OF MESENCHYMAL STEM CELLS BY REPRESSING E2F3

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Use of mesenchymal stem cell (MSC) has emerged as a potential new treatment for therapy to myocardial infarction; however, the poor viability of MSCs after transplantation critically limits the efficacy of this new strategy. MicroRNA-210 (miR-210) is induced by hypoxia and has an important role in cell survival under hypoxic condition. But role of miR-210 in regulation of MSC survival have yet to be delineated. Prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG) treatment increased hypoxia inducible factor-1 (HIF-1) protein level and expression level of miR-210 in MSCs, respectively, by western blot analysis and real time-PCR. To investigate effect of miR-210 in HIF-1 - induced MSC survival, MSCs were treated with DMOG, in absence or presence overexpression of miR-210 mimics. Enforced expression of miR-210 significantly inhibited survival of MSC and induced cell cycle arrest in G0/G1, compared with control miRNA. We identified an E2F family member E2F3 as targets of miR-210 through assessed activity luciferase reporter construct containing the 3′ UTR of E2F3 and expression of E2F3. Transcription factor E2F3 regulates the progression of cell cycle, influence on cell proliferation and survival. The cell transfected with siRNA against E2F3, cell death and cell cycle arrest was increased. These data demonstrate that miR-210 have important role in survival of MSCs under hypoxic response.

Poster Board Number: 2466

A NOVEL MICRORNA DIFFERENTIATES MESENCHYMAL STEM STEMS INTO ENDOTHELIAL LIKE CELLS BY MODULATING GSK-3β/β-CATENIN SIGNALING

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Bone marrow-derived mesenchymal stem cells (MSCs) possess multi-lineage differentiation potentials and have the ability to repair and rebuild injured vessels. MicroRNAs (miRNAs) are non-coding RNA molecules that regulate a subset of target genes involved in cell differentiation and specific cell function through posttranscriptional modulation. A number of studies have demonstrated that GSK-3β plays a critical role in several cellular processes, such

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as differentiation, growth, and apoptosis. Here, we described a novel miRNA that were identified by cloning from human MSC and cancer cell. Among these novel miRNAs, we identified binding site of 3 novel miRNA within GSK-3β 3′ UTR through sequence based analysis. Using luciferase assay, we identified one specific novel miRNA is direct target GSK-3β. Immunoblot analyses indicated that GSK-3β protein expression was dramatically decreased in hMSCs after transfection with this miRNA. To investigate the role of novel miRNA in differentiation, MSCs was transfected with novel miRNA mimic. Overexpression of miRNA led to a differentiated phenotype that was similar to that induced by SB216763 in human MSCs. The transfected cells showed a strong increase of expression of endothelial-specific markers (CD31, eNOS, VE-cadherin, VCAM-1, and VEGF-R2) and functional behavior of endothelial cells such as a formation of capillary-like structure. The differentiation of MSCs into endothelial-like cells by the treatment novel miRNA mimic was associated with GSK-3β/β-catenin signaling. In conclusion, these results indicate that novel miRNA regulate differentiation into endothelial cells by inhibition of GSK-3β/β-catenin signaling pathway. Our work suggests amount miRNA greatly relatives in MSCs and their differentiation into various fate.

Poster Board Number: 2468

THE INFLUENCES OF NONYLPHENOL ON HUMAN AMNIOTIC FLUID-DERIVED STEM CELLS

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The aim of this study is to explore the influences of Nonylphenol (NP) on human amniotic fluid-derived stem cells (AFSCs). Amniotic fluid samples were obtained from pregnant women by amniocentesis performed between 16 and 20 weeks of gestation for fetal karyotyping. AFSCs were isolated by two-stage culture protocol and then treated with NP (10, 50, 100, 200μM) for 24, 48, and 72 hours, respectively. The effect of NP on the proliferation of AFSCs was determined by trypan blue dye exclusion assay. The total number of viable cells was calculated by microscopy. RT-PCR and qPCR were used to assess the Oct4, Nanog, and Sox2 expressions of AFSCs. The viability of AFSCs decreased proportional to the increasing dosage or the length of incubation when they were treated with NP. In addition, the higher dosage of NP and the longer exposure of NP, the stronger Oct4, Nanog, and Sox2 gene expressions were found. When treating AFSCs with 90μM NP by 24 hours and 48 hours respectively, the levels of Oct4, Nanog, and Sox2 gene expressions on AFSCs were 1.1 and 2.2 for Oct4, 1.2 and 2.6 for Sox2, and 1.0 and 2.4 for Nanog respectively. These results indicated that NP might influence the process of cellular differentiation or organgenesis during fetal development.

Poster Board Number: 2470

LIN28 AFFECTS PROLIFERATION, DIFFERENTIATION AND REDUCES SENESCENCE OF HUMAN BONE MARROW DERIVED MESENCHYMAL STEM CELLS

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Studies have only recently begun to evaluate the role of miRNAs in multipotent bone marrow stromal cells/mesenchymal stem cells (MSC). Analysis of deep sequencing miRNA expression profile and measurements using real time PCR revealed high expression of let-7 family members in MSC. However, the study on a single miRNA level is difficult due to their redundancy and sequence identities with mature miRNAs commonly differing in a single nucleotide. The pluripotency factor Lin28 is a RNA-binding protein necessary and sufficient to block microprocessor-mediated cleavage of pri-let-7 miRNAs. We indirectly studied the role of let-7 in human MSC by lentiviral-mediated over-expression of Lin28. Similar to what has been described in fibroblasts, MSC engineered to over express Lin28 showed strong reduction of senescence *in vitro* as evaluated morphologically. In agreement with findings in embryonic stem cells, we observed that Lin28 induces a shift in cell cycle from S phase to G2, without affecting the percentage of quiescent cells (G0/G1), as compared to MSC transduced with a control lentiviral vector. Surprisingly however, this led to a slight, but significant decrease in proliferation of MSC by Lin28, dependent on the level of expression of the transgene. Of note, this mild reduction of proliferation mediated by Lin28 only occurs during 8-10 days after transduction. Thereafter, unknown compensatory mechanisms reestablish cell growth to control levels. When cultured in osteogenic medium for 14 days, MSC that over-expressed Lin28 showed 25% more calcium precipitation. When cultured in adipogenic medium, MSC that over-expressed Lin28 had increased triglyceride content and expression of adipogenic markers ppar-gamma and fabp4, as compared to controls. These findings show that Lin28 enhanced the differentiation potential of MSCs and reduced cellular senescence.

Poster Board Number: 2472

ELEVATION OF DIFFERENTIATION POTENTIAL OF HUMAN MESENCHYMAL STEM CELLS BY DIRECT DELIVERY OF RECOMBINANT CELL-PENETRATING PEPTIDE-COACTIVATOR-ASSOCIATED ARGININE METHYLTRANSFERASE 1 (CPP-CARM1) PROTEIN

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Introduction: Human mesenchymal stem cells (hMSCs) that have been used for clinical application have a limited potential of proliferation and differentiation, as sub-culturing *in vitro*. In order to overcome these obstacles, several delivery systems of biological proteins have been introduced recently in the experimental or clinical research field, and it may have also a merit for avoiding genomic manipulation that may cause tumor formation and other unexpected outcomes. In present study, we have applied a novel protein delivery system to elevate the functional potential of hMSCs through histone modification by methylation of histone arginine residues using cell-penetrating peptide coactivator-associated arginine methyltransferase 1 (CPP-CARM1) protein. Materials and Methods: Production of the CPP (R7)-CARM1 protein were performed by cloning using pET protein expression vector, and it was purified through 6x His-taq conjugated in c-terminal. This CPP-CARM1 was treated to cultured bone marrow (BM)-, adipose (AD)-hMSCs. The existence of CPP-CARM1 in the nuclei was analyzed by immunocytochemistry using anti-6x His-taq antibody. Also, in order to confirm functional activity of CPP-CARM1, we performed immunocytochemistry and Western blot analysis using anti-Histone H3 asymmetric dimethyl R17 (H3R17di-me) antibody. Finally we compared the efficiency of adipogenic and osteogenic differentiation of hMSCs by the histone methylation wity CPP-CARM1 protein through experiments such as Oil Red O and Alizarin Red S staining and realtime-RT-PCR. Results: CPP-CARM1 was localized in both cytoplasm and nuclei of hMSCs at 6 hr after protein treatment and remained in nuclei only at 12 hr. In groups treated with CPP-CARM1, di-methylation of histone H3R17 were more increased, compared to control group, and the higher levels of methylation were maintained at 12hr and 24hr after treatment. The

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level was lowered at 48 hr. In addition, treatment of CPP-CARM1 protein improved the efficiency of differentiation and it was confirmed by elevating Oil Red O and Alizarin Red S staining as well as expression levels of each lineage related genes by realtime-RT-PCR. Conclusion: We established a direct delivery system of CARM1 protein using cell-penetrating peptide (R7), and resulted in successful delivery into the nuclei of hMSC. Also, we found that treatment of CPP-CARM1 improved the differentiation efficiency of hMSCs into adipogenic, and osteogenic lineage. Therefore, our results suggest that this system may be a valuable tool to complement the weak point of hMSCs in clinical applications.

Poster Board Number: 2474

A PROSPECTIVE CELL SURFACE MARKER FOR HUMAN MESENCHYMAL STEM CELLS THAT READILY DIFFERENTIATE INTO CARDIOMYOCYTES

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Myocardial infarction caused by chronic atherosclerosis of the coronary artery could induce heart failure with high mortality. Because myocardium has very limited ability of regeneration, heart transplantation is currently considered to be the most effective treatment for severe heart failure. In the case of patients with some remaining heart functions, ventricular assist devices can be used to complement their lost functions. For severe patients who need to replace their heart, artificial hearts are used in order to bridge the time to heart transplantation for a limited period of time. However, at present these devices cannot be used forever and improved long-time antithrombogenicity, biocompatibility, reliability and durability are needed. On top of that, limited number of donor for heart transplantation causes serious issues of this methodology. Recently, stem cell therapy is expected to be the alternative regenerative medicine for heart failure. In addition to pluripotent embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, mesenchymal stem cells (MSCs) have been shown to differentiate into various cells including bone, cartridge, fat, neuron, skeletal muscle, and cardiac muscle lineages *in vitro*. Recent reports suggested that adult bone marrow-derived MSCs augment the recovery of cardiac function after ischemia *in vivo*. The effects induced by MSCs are generally temporary and limited. Because these stem cells show beneficial effects without long-time engraftment, the effects of these stem cells are considered due to paracrine effects of various cytokines secreted by injected stem cells. The successful differentiation of transplanted cells *in vivo* was mainly due to cell fusion of the injected cells with differentiated host cells. Thus, effective differentiation *in vitro* followed by successful engraftment is crucial for more practical application of MSCs for heart failure patients. In this study, we surveyed cell surface markers of human MSCs of which expression correlates with their cardiomyogenic differentiation ability. Various MSCs derived from human bone marrow or adipose tissues were used in this study. To analyze cardiomyogenic differentiation ability, GFP-labeled human MSCs were co-cultured on mouse embryonic cardiomyocytes *in vitro*. After 10 days culture, GFP-positive beating cells differentiated from human MSCs were counted. The differentiated cardiomyocytes were further evaluated by immunofluorescent staining with cardiomyocyte-specific antibodies against cardiac troponin T, α-actinin, and connexin-43. Among various cell surface markers tested, the expression of N-cadherin on MSCs showed good correlation with the differentiation abilities into beating cardiomyocytes. Our data suggested that N-cadherin could be a prospective cell-surface marker of human MSCs that have higher differentiation abilities into cardiomyocytes.

Poster Board Number: 2476

CHARACTERIZATION OF HUMAN BONE MARROW STROMAL/STEM CELLS UNDERGOING CARDIAC DIFFERENTIATION

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Cardiovascular diseases are the leading cause of death in North America. Heart tissue has no/limited capacity to regenerate; as a result, cardiomyocyte loss and the subsequent pathological remodeling of the cardiac tissue lead to heart failure after myocardial injury. In recent years, cell replacement approaches with a plethora of cell types have been investigated to mitigate these pathological effects and restore cardiac function in animal and preclinical studies. However, the outcome of these studies is variable and generally shows modest functional improvements, which implicate the necessity to identify "optimal" cell type and/or factors that enhance the regenerative capacity of cell transplants. Mesenchymal Stromal/Stem Cells (MSCs) have unique properties that make them good candidates for cell-based therapies. Here we have investigated the effect of 3D environment (collagen scaffold) and cadiomyogenic priming (via co-culturing with primary cardiomyocyte) on biological and functional properties of bone marrow-derived human MSCs using an *in vitro* micro-engineered heart model. We have shown that both 3D environment and cardiogenic priming enhance proliferative activity of hMSC assessed by cell cycle profile and colony forming capability. Primed hMSCs injected onto engineered heart tissue (EHT) neither interfere with nor contribute to the electrical property of EHT though improve the force of contraction in EHT. We have shown that while 3D environment enhances the cardiomyogenic inductive effect of co-culture, evaluated by the expression of cardiac-specific genes by primed hMSCs, covalent immobilization of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) on scaffolds helps primed MSCs to sustain the acquired cardiac lineage phenotype. The result of the latter finding may lead to the potential development of hMSC grafts that could be directly applied to the site of myocardial injury.

Poster Board Number: 2478

TENASCIN C AND WNT-7A MODULATE NEURONAL DIFFERENTIATION OF HUMAN BONE MARROW DERIVED MESENCHYMAL STEM CELLS

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Adult human mesenchymal stem cells are generally restricted in their differentiative potential to the tissue where they reside. However, previous studies in our laboratory reported brain-derived neurotrophic factor (BDNF)/neurotrophins (NT)/retinoic acid (RA) triggered a transdifferentiation of human mesenchymal stem cells (hMSCs) toward neuronal progenitor-like cells. Extracellular matrix (ECM) protein, tenscin-cytotactin (Tn-C), and Wnt-7a further showed a trans-differentiation and somatic plasticity phenomena in cell culture. The BDNF/NT/RA triggered neuronal differentiation of hMSCs resulting in increased mRNA expression of neuronal markers such as

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nestin, microtubule associated protein-2 (MAP2), glial fibrially acidic protein (GFAP), βIII tubulin and neuron growth factor (NGF). The addition of Tn-C continued enhancing mRNA expression of MAP2, βIII tubulin and NGF. Wnt-7a found to significantly increase MAP2, βIII tubulin, NGF, and Myelin Basic Protein (MBP). Incorporation with Wnt-7a and lithium in culture, hMSCs found to express synapsin1 (Syn1). The induction of Syn1was inhibited after adding Wnt inhibitors. Moreover, Wnt7a triggered formation of cholinergic, dopaminergic, GABAergic and serotonergic neurons. In conclusion, ECM such as tenscin C and Wnt-7a, may play a functional role in modulating neurogenesis by MSCs. Canonical Wnt7a is crucial to synapse formation and neuron type determination in hMSCs. These findings suggest that the combined use of Tn-C, Wnt-7a, BDNF/NT/RA, and MSCs offers a novel cell therapy in clinic in the future. Keywords: mesenchymal stem cell, Wnt-7a, neurogenesis, synapsin, tenascin C, transdifferentiation

Poster Board Number: 2480

OPTIMIZATION OF CULTURE CONDITION OF HUMAN UMBILICAL CORD BLOOD-DERIVED MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSCs) have several features of pluripotency, paracrine effects, and immunosuppressive properties, which lead to clinical trial implementation. It is important to increase the proliferation rate of the MSCs which is essential of mass production of MSCs for clinical use. In this study, several factors were tested to up-regulate the proliferation and their several effects on MSCs were evaluated. It has been reported that several factors increase the proliferation rate of adherent mammalian cells. Among them, we selected the most effective factors on umbilical cord blood-derived MSCs (UCB-MSCs) and tested the effects on the proliferation. Calcium, hypoxia, and several extracellular matrix (ECM) proteins like fibronectin and thrombospondin-1 (TSP-1) were tested using proliferation assay with UCB-MSCs. Optimal concentration of calcium was determined with concentration range of 0~3mM without the change of differentiation capacity. The effects of individual factors were different among UCB-MSC batches due to the lot variations. To diminish different effects of factors according to the batch of MSCs, their combinations were conducted and investigated to maximize the proliferation of MSCs. Proliferation of UCB-MSCs was increased 2-fold in individual treatment and maximum 6-fold in combined ones. Several characteristics of UCB-MSCs after treatment of factors were monitored to evaluate their properties. Their effects on the growth of MSCs were evaluated by proliferation assay. The differentiation of UCB-MSCs into several cell types including osteoblasts, chondrocytes and adipocyte were tested. Also, the surface markers of UCB-MSCs were monitored by FACS analysis, which all showed no critical difference in MSC properties. In conclusion, single or combinations of several factors were adjusted to up-regulate the proliferation, and their effects on the properties of UCB-MSCs were evaluated.

Poster Board Number: 2482

TRANSPLANTATION OF NEURAL PROGENITORS GENERATED FROM HUMAN PLACENTAL MESENCHYMAL STEM CELLS INCREASE DOPAMINE UPTAKE AND ALLEVIATE ASYMMETRIC MOTOR BEHAVIOR IN THE RAT MODEL OF PARKINSON'S DISEASE

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Parkinson's disease (PD) is a degenerative disorder characterized by a progressive loss of dopaminergeic neurons in the mesencephalic substantia nigra. Symptoms of locomotive dysfunctions of the illness were relieved by pharmacological treatments but the treatments accompany side effects if administered for a long term. Alternatively, fetal mesencephalic tissues were transplanted with some success, however, the uitilization of human fetal tissue caused ethical and religious debates as well as difficulties in availability. Recently developed stem cells are a group of cells that are capable of self-renewal and differentiation and thus are considered as promising candidates for the replacement of human tissues or organs. We previously isolated 17 independent mesenchymal stem cells from the first trimester human placenta (fPMSC) and successfully differentiated the cells neural progenitor cells. When transplanted into the striatum of the 6-OHDA-induced PD animal models, the *in vitro*-differentiated neural progenitors differentiated into dopaminergic neurons, normalize the asymmetric rotational behavior of the animals, and increased the uptake of 18F-fluorodopa in the ipsilateral brain as determined by PET. These results suggest that the *in vitro*-differentiated neural progenitors may be as effective as the terminally differentiated cells for the cell replacement therapies.

Poster Board Number: 2484

A NEW METHOD OF THE DIFFERENTIATION OF MESENCHYME STEM CELLS FROM HUMAN PLACENTA INTO NEURON-LIKE CELLS *IN VITRO*

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BACKGROUND AND AIMS. Human placenta may contain plenty of mesenchymal stem cells (MSCs), which can be differentiated into neuron-like cells. At present, it is always used conditional medium containing some specific growth factors to induce MSCs. This study was aimed to explore a new way to the differentiation of MSCs from human placenta into neuron-like cells *in vitro*. METHODS. MSCs from human placenta were isolated and purified by cell culture. The third passage of MSCs was incubated with DMEM containing 20%FBS for 24 hours. Then add Diff-1 medium supplemented with 0.2%FBS, bFGF and EGF. Continue culture for 24 hours. Replace medium with fresh Diff-1 medium supplemented with 0.2%FBS,200ug/ml (protein) of CNS tissue extracts. Continue culture for 6 days. Replace medium and add to RA and Shh .Continue culture for 3 days. Finally, replace medium with fresh Diff-1 supplemented with 10%FBS and add BDNF, GDNF and IGF-II for 7 days. Cell morphology was observed. Nerve cell markers were detected by immunohistochemistry and ELISA assays. RESULTS. The adhesive cells gradually formed flat monolayer cells, parallel arranged or whirlpool grew, with uniform morphology after 2 passages. Compared

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with control, the cells expressed CD90, CD44, CD73, CD29, et al. But not expressed CD45 and CD34. After induce culture, NSE,Nestin,GFAP,NF-M, GAP-43 could be expressed by immunohistochemical staining test. NT3 ,NT4 and BDNF were expressed in the supernatant by ELISA kit analysis. CONCLUSION. The result demonstrated that MSCs from human placeta can differentiate into neuron-like cells, which add the protein of CNS tissue extracts to medium.

Poster Board Number: 2486

SINGLE CELL ISOLATION ELUCIDATES HETEROGENEITY WITHIN THE HUMAN MESENCHYMAL STEM/PROGENITOR CELL COMPARTMENT

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Background: Multi-potent mesenchymal stromal cells (MSCs) are self-renewing cells with the ability to differentiate into osteocytes, chondrocytes, adipocytes, myocytes and also neuron-like cells and hepatocytes. Because of their multi-potency and low immunogenicity, MSCs are considered a potential candidate for clinical applications including cartilage reconstitution, and the therapy of inherited diseases such as osteogenesis imperfecta. Traditionally, MSCs are isolated from primary tissue based on their capacity to adhere to a plastic surface. This isolation procedure is hampered by the unpredictable influence of co-cultured hematopoietic and other contaminating cells. To circumvent these limitations, several surface markers have been identified to facilitate the prospective isolation of MSCs. Results: Here we provide a high quality method using flow cytometry to isolate a pure population of clonogenic human MSCs based on the expression of low-affinity nerve growth factor receptor (LNGFR) and Thy-1. Multicolor cell sorting and CFU-F assays showed that mesenchymal stem cells were ~33,000-fold enriched in the LNGFR+Thy-1+ fraction. The LNGFR+Thy-1+ cells contained CFU-Fs at the frequency of one per six cells. Clonogenic assays of these cells revealed 3 distinct sub-populations within we have designated rapidly proliferating clone (RPC), moderately proliferating clone (MPC) and slowly proliferating clone (SPC). RPCs demonstrated robust multi-lineage differentiation and self-renewal potency. In order to validate these results we established 145 clonally derived LNGFR+Thy-1+ MSC populations. From the 46 RPC, 37 (80%) clones gave rise to all adipogenic, chondrogenic and osteogenic lineages. In contrast, only 6 (13%) clones of SPC demonstrated tri-lineage differentiation capacity. Surprisingly, 16 out of 51 SPC (31%) were only able to differentiate to bone or unable to differentiate at all. Additionally we noted the responsiveness of each clone type to adipogenic differentiation appears to be linked to its proliferative capacity and therefore growth kinetics may predict the stem-like potential hMSCs. From our data so far we concluded that RPC contained cells with most stem like qualities. In order to determine if any additional surface markers could distinguish between RPC, MPC and SPC we looked for a difference in expression of hMSCs markers between these different cell types. The most marked difference that we noted was moderate expression of CD106 in RPC following 6 weeks in culture (54.9%) compared to absent expression in MPC and SPC. To examine and define the phenotype among the CD106-positive cells, we perform secondary single cell sorting in CD106-positive and CD106-negative cells. Interestingly, the CD106-positive cells yielded high colony forming capacity. These results indicating that MSC compartment are a heterogeneous population that may be sub-divided into RPC, MPC and SPC based on growth kinetics. Conclusions: We describe a method to prospectively isolate a pure population of potent MSCs based surface expression of LNGFR and Thy-1. This population is composed of sub-populations which differ in their replicative ability and plasticity. We provide strong evidence for biological heterogeneity within an MSC population.

Poster Board Number: 2488

NEUROPROTECTIVE EFFECTS OF HUMAN UMBILICAL CORD MESENCHYMAL STEM CELLS IN AN ANIMAL MODEL OF PARKINSON'S DISEASE

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Human mesenchymal stem cells (hMSCs) are reported to possess immunomodulatory properties and secrete various neurotrophic factors. We have demonstrated that inflammation plays a crucial role in Parkinson´s Disease (PD) progression. In this study, we evaluated whether the transplantation of hMSC obtained from umbilical cord (UC) has a neuroprotective effect in a rat PD model. To address the neuroprotective effect of these cells, we implanted undifferentiated hMSCs into the sustantia nigra pars compacta (SNc) of rats bearing a lesion of the nigrostriatal pathway induced by the intrastriatal injection of 6-hydroxydopamine (6-OHDA), a widely recognized rodent model of PD. The hMSCs were transplanted 7 days after 6-OHDA injection and animals were sacrificed 14 days later. Umbilical cord hMSC expressed markers characteristic of mesenchymal stem cells: CD 90 (+), CD 73 (+), CD 105 (+), CD 44 (+), CD 34 (-), HLA-DR (-), CD 14 (-) and CD 45 (-). Our results revealed that rats receiving hMSCs transplantation displayed significant preservation in the number of dopaminergic neurons in the SNc compared to control rats. However, rats receiving hMSC transplantation did not show a significant behavioural amelioration in a cylinder test compared with the control groups at the experimental time points analysed. Even though the animals did not receive an immunosuppressive treatment the inflammatory response against human transplanted cells was restricted to the implantation area. This results suggest that grafted hMSCs exert neuroprotective effects on dopaminergic neurons against the degeneration induced by 6-OHDA. The mechanisms underlying this effect remain to be clarified, although we favour the hypothesis that hMSCs have a neuroprotective effect through anti-inflammatory actions.

Poster Board Number: 2490

EFFICIENT LABELING OF HUMAN MESENCHYMAL STEM CELL WITH IRON OXIDE NANO PARTICLE FOR *IN VIVO* TRACKING

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Transplantation of adult Mesenchymal stem cells (MSCs) has shown promise for the treatment of several degenerative diseases. However, the inability of present strategies, to non-invasively track these cells after *in vivo* administration has been a significant roadblock to the clinical acceptance of many cellular therapies. This has motivated the development of non-invasive imaging techniques to track the transplanted cells. In this study, we sought to determine if bone marrow derived MSCs, labeled with in-house Iron Oxide Nano Particle (IONP) can retain their phenotype, proliferation and differentiation potential. This study was initiated after IRB clearance. Iron oxide nano particles of 7-10 nm size were prepared and were characterized for size, chemical composition using various techniques like Transmission Electron

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Microscopy, X-ray diffraction, Attenuated Total Reflection Fourier Transform Infrared and Dynamic light scattering. MSCs were isolated and expanded from human bone marrow, by using established protocols. At confluence, cells were labeled with IONPs. These IONP labeled cells were stained with Prussian blue stain to estimate the labeling efficiency and further passaged to determine cell proliferation and IONP retentions during expansion. After successful labeling of MSCs with IONP, the cells were investigated for any change in the surface marker expression by flow-cytometry; CD29, CD90, CD73, CD105, HLA I & II. The labeled cells were induced to differentiate into various cell lineages including osteoblasts, chondrocytes, adipocytes, cardiomyocytes and neurons. During induction, cells were observed carefully to see the change in phenotype i.e. cell morphology, matrix mineralization and cytoplasmic lipid vesicles formation. At the end of induction period, differentiated cells were characterized at transcript and protein levels by RT-PCR and immunofluoresence. Unlabelled MSCs served as controls in all the experiments. IONPs labeled cells were also investigated for their tracking ability using non-invasive technique i.e. MRI. Labeled MSCs were injected into rat models of Myocardial infarction by tail vein as well as direct in the infarct tissue. After injection MRI was done after 24 hrs and 3 weeks to track the IONP labeled cells. IONPs labeling did not alter the surface marker profile, proliferation rate, differentiation potential of MSCs. IONPs were retained in the MSCs till 4th passage. *in vivo* study also revealed the tracking efficiency of IONPs labeled MSCs for long duration up to three weeks in the infracted myocardial tissue. This study revealed that MSCs can be labeled efficiently with this novel IONP without any change in stemness, expansion and differentiation potential. This in-house developed IONP may be a useful tool for non-invasive tracking of transplanted MSCs.

Poster Board Number: 2492

CORD BLOOD PLATELET GEL FOR GMP PRODUCTION OF HUMAN MESENCHYMAL STEM CELL: AN INNOVATIVE, SAFE AND ANIMAL-FREE SUBSTITUTE FOR CLINICAL APPLICATIONS

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Human mesenchymal stem cells (MSC) are used in clinical trials for their immunomodulation ability and more recently for their tissue regeneration properties. Their production has to be done under GMP conditions and crucial is the definition of the optimal cell culture components that could impact on cell yield and differentiation. Routinely, fetal bovine serum (FBS) is used for MSC culture but its associated risks (e.g. disease transmission and bovine protein sensitization) are well known. An interesting and safe alternative component is cord blood (CB) platelet gel (PG) that we have recently produced, validated and patented. This CB-PG contains higher concentration of some growth factors such as VEGF in comparison with its peripheral blood counterpart (1803.5 ± 788.5 pg/mL vs 250 ± 120 pg/mL, respectively) and lower levels of some other factors including TGFbeta1 (6865.6±2668.5 pg/mL vs 11250 ± 6796). In order to test the ability of CB-PG releasate to replace FBS, adipose MSC were cultured in standard conditions in the presence of 20% CB-PG batroxobin-induced releasate or 20% FBS. During and after the culture, adipose MSC in the presence of CB-PG show a higher cell expansion rate compared to FBS cultures. In addition, adipose MSC cultured in CB-PG releasate were more prone to differentiate toward chondrogenic, adipogenic and osteogenic cell lineages. Moreover, adipose MSC showed a higher rate of CFU-F. The telomerase activity was normal when compared to the HELA cells. These interesting findings prompted us to investigate the proteomic profile of CB-PG using four different approaches: ELISA (6 factors; R&D Systems, Minneapolis, MN, USA), multi-analyte profiling platform (189 factors; RBM, Austin, TX, USA), SELDI-ToF-MS (Surface Enhanced Laser Desorption/Ionization-Time of Flight-Mass Spectometry) and Reverse Phase HPLC coupled with SDS PAGE. Some analyses are still ongoing but the preliminary results show that CB-PG can be a valid alternative to FBS when MSC have to be produced for angiogenic-mediated tissue repair. This peculiarity could be explained by extending the properties of CB stem cells as part of perivascular/endothelial compartment to CB-PG.

Poster Board Number: 2494

SOX2 CONTROLS THE DIFFERENTIATION OF HUMAN UMBILICAL CORD BLOOD-DERIVED MESENCHYMAL STEM CELLS BY DIRECT REGULATION OF DKK1 AND GSK3-β PHOSPHORYLATION

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SOX2 is a well-known core transcription factor in embryonic stem cells (ESCs) and one of the reprogramming factors to make induced pluripotent stem (iPS) cells. The function of SOX2 has been researched mainly in neural stem cells. Recently, SOX2 expression in human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) has been reported by us and others. However, the mechanism of SOX2 in stem cells has not been known well. To identify the molecular mechanism of SOX2, inhibition study was performed in hUCB-MSCs. SOX2-inhibited hUCB-MSCs showed severe proliferation defects. Additionally, the adipogenic differentiation of SOX2-inhibited hUCB-MSCs decreased. In contrast, the osteogenic and chondrogenic differentiation increased. After SOX2 inhibition, the phosphorylation of GSK3-β at serine 9 increased as a consequence of DKK1 depression in SOX2-inhibited hMSCs. SOX2 bound to the promoter region of DKK1 and has a positive regulatory role in DKK1 transcription. Ectopic treatment of DKK1 in SOX2-inhibited hUCB-MSCs recovered the differentiation deformities but could not abrogate the cell proliferation defect. These results show that SOX2 directly regulates DKK1 expression and, as a consequence, regulates phosphorylation of GSK3-β during differentiation of hUCB-MSCs. Taken together, SOX2 is critical to the differentiation of stem cells was evaluated.

Poster Board Number: 2496

HUMAN MENSTRUAL BLOOD-DERIVED MESENCHYMAL CELLS AS A CELL SOURCE FOR RAPID AND EFFICIENT NUCLEAR REPROGRAMMING

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Induced pluripotent stem cells (iPSC) were originally generated by forced ectopic expression of four transcription factors - Oct4, Klf4, Sox2 and c-myc - in adult fibroblasts. However, the efficiency of iPSC recovery is extremely low, varying from 0.01 - 0.1%, and the nuclear reprogramming takes about 20 days to be accomplished. We reasoned that adult cells showing basal expression of core embryonic stem (ES) cell regulator genes, such as Oct4, Sox2, Klf4 or Nanog, could be a better cell source for reprogramming. Men-

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strual blood-derived mesenchymal cells (MBMC) are multipotent cells that show detectable transcripts and protein levels of some of the core ES regulators, such as Oct4, Sox2 and Klf4. The aim of this study was to determine whether reprogramming efficiency could be increased by using MBMCs as a cell source to generate iPSC. In order to reprogram these cells, two rounds of transduction were made using a mixture of 3 recombinant retroviruses expressing the coding region of Oct4, Sox2 and Klf4 genes. Twenty-four hours after the second round of transduction, MBMCs were plated on Mitomicin C-inactivated mouse embryonic fibroblasts (iMEF). Nuclear reprogramming was detected as early as 5 days post-transduction by the appearance of cells with high nucleus/cytoplasm ratio, and colonies begun to appear after 7 days. At day 15, colonies were picked-up and expanded for characterization. Most of the clones were morphologically identical to ES cells and positive for alkaline phosphatase (AP). Some of the clones expressed the cell surface pluripotency marker SSEA-4 and TRA-1-60 and the transcription factors Nanog and Oct4. RT-PCR analysis detected the transcripts of the pluripotency marker genes Oct4, Sox2, Klf4, Nanog, Dnmt3b, Nodal, Tdgf and hTert and qRT-PCR assays confirmed robust expression levels. They are capable of forming embryoid bodies and to differentiate *in vitro* into cells of the three germ cell layers. Nonetheless, our results showed that the reprogramming was faster and with efficiency around 2 - 5%, even in the absence of c-myc. To date, this is the first study showing MBMCs as a cell source for nuclear reprogramming.

Poster Board Number: 2498

REDUCED PROLIFERATION AND DIFFERENTIATION EFFICIENCY AFTER OCT4 KNOCK-DOWN IN PLACENTA DERIVED MESENCHYMAL STROMAL CELLS

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Mesenchymal stromal cells (MSCs) are adult multipotent cells able to differentiate toward mature mesodermal lineages. Despite years of investigation, little is known about the molecular mechanisms underlying the stemness state in these cells. The Pou domain-containing transcription factor Oct4 is a well established master regulator of pluripotency in the inner cell mass of the embryo as well as in embryonic stem cells. While it has been shown that the Oct4 gene is inactivated through a series of epigenetic modifications following embryonic development, recent studies have detected Oct4 mRNA and protein in a number of somatic stem cells, mesenchymal stromal cells and tumor cells. These observations suggest that Oct4 may have a function in maintaining self-renewal and stemness in these cells. We employed an RNA interference approach to determine whether Oct4 gene expression is important for maintaining stemness and self-renewal in the placenta-derived mesenchymal cells. Using lentiviral constructs which constitutively give rise to siRNAs targeted against Oct4 mRNA, about 80-90% knock-down of the Oct4 transcript levels were obtained. These cells showed a significant reduction in proliferation as verified by Ki67 immunoassays (about 25%) and by Brdu incorporation assays (23.5%). When these cells were induced to differentiate into osteocytes and adipocytes, they showed a delayed kinetics and reduced efficiency as compared to wild-type cells. The molecular mechanisms underlying the reduced proliferation and reduced differentiation efficiency are currently being investigated. These results suggest that Oct4 may have a function in stemness and self-renewal of mesenchymal stromal cells derived from the placenta.

Poster Board Number: 2500

IGF-1 INDUCES PROLIFERATION AND MYOGENIC DIFFERENTIATION OF STROMAL STEM CELLS FROM HUMAN UMBILICAL CORD TISSUE

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Progressive muscular dystrophies are a group of disorders characterized by progressive and irreversible muscle degeneration for which there is no therapy. Human umbilical cord tissue has been considered as an important source of stromal stem cells (SSC), which are able to differentiate into distinct cell types. However, there is limited information concerning favorable conditions that induce differentiation of SSC into muscle cells. It has been demonstrated that insulin-like growth factor-1 (IGF-1) promotes survival, proliferation, migration and myogenic differentiation of muscle progenitor cells, but its effects on stem cells has not been reported. Here we investigated, for the first time, if IGF-1 is capable of inducing the proliferation and myogenic differentiation of SSC from human umbilical cord tissue *in vitro*. Our results showed that IGF-1 induces SSC proliferation. Moreover, we observed that SSC undergo activation of myogenic regulatory factors (MRFs), followed by extensive muscle differentiation with a transient increase of MyHC expression when exposed to IGF-1. In summary, our data indicate that IGF-1 increases proliferation and myogenic differentiation of SSC. These results may have important applications for future therapies in patients with different forms of muscular dystrophies. Supported by CEPID, INCT and CNPq.

Poster Board Number: 2502

NURR1 AND GDNF GENE PRODUCTS SYNERGISTICALLY INDUCE DOPAMINERGIC DIFFERENTIATION OF HUMAN UMBILICAL CORD MATRIX STEM CELLS

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Dopaminergic transcription and neurotrophic factors support dopamine signaling in embryonic development and have shown their potential to accelerate differentiation of appropriate cell sources toward dopaminergic fate. Here, we report the synergistic effects of Nurr1 transcription factor and GDNF neurotrophic factor on neuronal differentiation of human umbilical cord matrix stem cells. We isolated these mesenchymal stem cells from human Wharton's Jelly and cultured in DMEM supplemented with serum and insulin-transferin-selenium (ITS) for a week before characterizing them for expression of stem cell markers. The cells largely expressed CD29, CD44 and SH2 whereas CD34 and CD45 expression could not be detected. Next, we transduced these cells using recombinant lentivirus vectors harboring either Nurr1/GFP (pLV-Nurr1-GFP), or GDNF/Jred coding sequences (pLV-GDNF-Jred). Following RT-PCR detection of transgene overexpression in transduced cell populations, we cultured the cells in defined medium supplemented with sonic hedgehog and FGF8. Compared to control cells transduced with empty vectors, the test cells displayed neuronal morphology with distinct neurite formation. Furthermore, we detected expression of dopamine synthesizing enzymes TH and AADC in the transduced test cells. However, the cell group that was transduced with both recombinant virus

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stocks and so co-expressed Nurr1/GDNF subsequently expressed higher levels of TH and AADC compared to when either virus stock was applied. Our data suggest that Nurr1 and GDNF synergize to accelerate dopamine signaling pathways and that their strong co-presence can direct a substantial proportion of the transduced test cells toward dopaminergic fate.

Poster Board Number: 2504

GROWTH/DIFFERENTIATION FACTORS MAINTAIN TENDONGENIC CHARACTERISTICS OF HUMAN PERIODONTAL LIGAMENT DERIVED CELLS IN CULTURE

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Objective: Native periodontal ligament-derived cells (PDLCs) constantly express both osteogenesis and tendongenesis-related genes, but when passaged in culture, PDLCs have a tendency of de-differentiation. Although it is well known that dexamethasone or BMP2 can induce/enhance osteogenic characteristics of PDLCs, little is known about effective factors that can induce/enhance tendongenic characteristics of PDLCs. In this study, we examined whether GDFs can enhance or maintain tendongenic characteristics of human PDLCs being expanded in culture during several passages for potential use as a cell source for tissue engineering of tendons and ligaments. Methods: Human PDLCs were obtained from extracted teeth, digested with collagenase, and the isolated cells subcultured until passage 6 for experiments herein. PDLCs from passages 3-6 were cultured until 70% confluency and subsequently placed in serum-free media with or without various concentrations of recombinant human GDF5 (100 ng/ml) and/or GDF7 (1-200ng/ml). Then, these treated cells were evaluated using cell proliferation (WST8), alkaline phosphatase (ALP) activity, reverse transcription -polymerase chain reaction (RT-PCR) and quantitative real-time PCR to determine the expression of tendongenic- and osteogenic-related genes. Human mesenchymal stem cells (MSCs) and dental pulp-derived cells (DPCs) were used as controls. Results: GDFs reduced ALP activity in cultured PDLCs, but its effect was reduced during passages. In contrast, ALP levels in MSCs and DPCs were increased by treatment with GDF5. Consistent with this result, the expression of osteogenic-related genes was down-regulated in GDFs-treated PDLCs, while its expression was up-regulated in MSCs or DPCs. On the other hand, the expression of tendongenic-related genes was up-regulated in GDFs-treated PDLCs. In particular, tenomodulin expression (a late marker of tendongenesis) was maintained in GDFs-treated PDLCs even at passage 6, contrasting with its disappearance in non-treated PDLCs. This suggests that GDFs stimulation rescued tendongenic characteristics in de-differentiated PDLCs. We are currently investigating additional inducible tendongenesis characteristics by GDFs in cultured PDLCs.

Poster Board Number: 2506

NOVEL POPULATION OF NESTIN POSITIVE PROGENITORS IN RAT ADULT PROSTATE DIFFERENTIATES INTO INSULIN PRODUCING CELLS FROM SGL1: A BIOACTIVE COMPONENT OF ENICOSTEMMA LITTORALE

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By 2010 the number of people with Diabetes Mellitus has reached upto 350 million worldwide. Major hurdles in treatment of diabetes are the current treatment of exogenous insulin supply which is not fully capable of achieving tight control over glucose regulation, leading to long-term complications. Recent success in islet transplantation-based therapies for diabetes mellitus and storage of pancreatic islets have motivated sprouting efforts to develop renewable sources of islet replacement tissue. A major impediment to our understanding of the biology of stem cells is the inability to distinguish them from their homing niche and multipotent differentiating property which makes them usable for islet replacement therapy. We made efforts to make use of association of stem cells with adult prostate to isolate a novel population of prostatic stem/ progenitors that express higher levels of nestin, a neuroendocrine protein and vimentin, mesenchymal stem cell marker, enabling a transformative link for endocrine cell differentiation. Enicostemma littorale, an antidiabetic medicinal Indian herb recently reported from our lab to have islet neogenic potential from various multipotent stem/ progentiors. In present study we analyzed selected population from prostate tissue which has a basal phenotype of fibroblastic mesenchymal with high proliferative and mulitpotential nature, as determined by expression of Nestin, Vimentin, SMA proteins by confocal microscopy and has a greater ability to form islet like clusters in-vitro upon differentiation with SGL-1 bioactive component from Enicostemma littorale. Furthermore the presence of dithizone staining and immunopositivity for islet hormones in post differentiated prostate progenitors suggests that these cells share common regulating pathways with pancreatic stem cells during developmental fate in organogenesis. Our study provides an evidence for distinct source for neuroendocrine progenitors which can be isolated and used for diabetic patients who are undergoing prostectomy

Poster Board Number: 2508

FGF2 BUT NOT WNT3A OR BMP2 INHIBIT MOUSE MESENCHYMAL STEM CELL DIFFERENTIATION BY INDUCING TWIST2 AND SPRY4 AND BLOCKING ACTIVATION OF EXTRACELLULAR REGULATED KINASE

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Cell signaling pathways that regulate adipogenic, chondrogenic, and osteogenic differentiation of mesenchymal stem cells (MSCs) are well described but those that maintain cells in an undifferentiated state remain largely unexplored. Previously we reported that FGF2 reversibly inhibited multilineage differentiation of primary mouse MSCs. Herein we identify a unique compliment of signaling proteins expressed in MSCs that are dynamically regulated by FGF2 and contribute directly to its inhibitory effect on cell differentiation. FGF2 significantly induced expression of Twist2, Sprouty4 (Spry4) and Cyclin D1 (Ccnd1) in MSCs and down regulated expression of

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soluble frizzled related receptor 2 (Sfrp2), runt-related transcription factor 2 (Runx2), and peroxisome proliferation activated receptor gamma (Pparg). In contrast, WNT3A induced expression of Twist but not Twist2 or Spry4 and bone morphogenetic protein 2 (BMP2) failed to alter expression of these mRNAs. Consistent with these findings, pre-treatment of MSCs with FGF2 blocked osteogenic and chondrogenic differentiation whereas WNT3A had only a partial inhibitory effect and BMP2 augmented osteogenic differentiation. Moreover, FGF2-induced changes in Spry4 mRNA and protein expression levels in MSCs were inversely correlated with the extent of extracellular regulated kinase 1 and 2 (ERK1/2) activation. Finally, immunostaining demonstrated that Twist proteins and Spry4 were co-expressed in a small subpopulation of MSCs and that FGF2 enriched for this subpopulation and also altered the sub cellular localization of both proteins. Collectively, these studies demonstrate that FGF2 modulates differentiation of MSCs by modulating expression levels of Twist2 and Spry4, the latter of which inhibits ERK1/2 activity.

Poster Board Number: 2510

THE ROLE OF TISSUE-RESIDENT MESENCHYMAL PROGENITORS IN THE DEVELOPMENT OF CARDIAC FIBROSIS IN MICE

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Abstract: Fibrosis is defined by excess deposition of extracellular matrix components including collagen, and is associated with a wide variety of pathological states in numerous different tissues. The functional implications of the development of fibrosis are well documented, but a thorough understanding of the cellular and molecular mechanisms underlying its progression is still lacking. Recent studies in skeletal muscle have identified a population of fibro/adipogenic progenitors (FAPs) and demonstrated their vital role in regulating both muscle regeneration and formation of fibrosis following muscle damage. Further data has supported the notion that of FAPs (CD31-:CD45-:alpha7-:Sca1+:PDGFRa+) are activated during regeneration in other tissues, including (but not limited to) adipose, skin, lung and bladder. We hypothesize that FAPs are present in the heart and may play an important role in development of cardiac fibrosis. Following enzymatic digestion of murine hearts, FAPs were isolated from the heart and expanded in culture. These cells possessed similar surface phenotypes to populations previously isolated from skeletal muscle. Following *in vitro* expansion we were successfully able to differentiate these cells toward adipogenic and fibrogenic lineages, as evidenced by Oil Red O and fibroblast marker staining (respectively), thus confirming them as FAPs. Using PDGRa-EGFP transgenic mice, we visualized the cardiac tissue distribution of these cells. We investigated the genetic profile of freshly sorted cardiac FAPS and found them to significantly upregulate several ECM-producing genes as compared to Lin-Sca1+PDGFRa-, Lin-Sca-, and Lin+ cell populations. We induced cardiac fibrosis in PDGFR-EGFP mice by injecting isoproterenol (100mg/kg/d) subcutaneously for 5 days, and isolated the heart 10 days later. ECM-producing genes were significantly upregulated as compared to FAPs from undamaged hearts. FAPs from muscle have been shown to express high levels of IL-6, which has been proposed as a potential mediator of their trophic role in regulating skeletal muscle regeneration. IL-6 has been suggested as an important signaling molecule mediating FAPs paracrine effect on tissue regeneration. Experiments on IL-6 KO mice demonstrated an increased HW/BW ratio as well as cardiac fibrosis at 6-weeks of age. Following isoproterenol-induced cardiac fibrosis, we observed increased fibrosis as compared to WT-controls, and significant proliferation of the FAP population evidenced by increased PDGFRa staining in heart sections. These results highlight FAPs important role in reparative processes following cardiac injury, and suggest they may be a key component for any strategies intended to augment cardiac regeneration.

Poster Board Number: 2512

ACTIVATION OF P130 AND E2F4 IS ASSOCIATED WITH INDUCTION OF WNT/B-CATENIN SIGNALING AND FORMATION OF THE P130/GSK3B/B-CATENIN COMPLEX IN MOUSE MESENCHYMAL STEM CELLS

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Signaling pathways, which regulate cell differentiation and self-renewal, function in cell cycle context. Proteins p130 and E2f4, members of the retinoblastoma protein (pRb) family/E2F transcription factor family, are the key elements in regulation of cell cycle and differentiation for majority of cell lines however their roles in somatic stem cells are still obscure. We demonstrate here that activation of the Wnt/β-catenin pathway in mesenchymal stem cells (MSC) by co-culture with A-549 cells or by lithium ions was associated with accumulation of active forms of the β-catenin, p130 and E2f4 but did not result in inhibition of cell cycle progression. The levels and phosphorylation patterns of p130, E2f4 and β-catenin in MSC did not change during cell cycle progression in contrast to control T98G glyoblastoma cells which accumulated differently phosphorylated forms of the p130 in quiescence and under active proliferation. During G0/G1 and S cell cycle phases in MSC E2f4 retained the ability to interact with p130, p130 and β-catenin physically bound each other, while Gsk3β bound and coprecipitated both p130 and β-catenin. Our results indicate that Wnt/β-catenin and pRb signal pathways may interact in MSC to form the p130/β-catenin/Gsk3β complex. Physiological relevance of such a complex may be associated with coupling cell cycle and differentiation in MSC related to their wide differentiation potential.

Poster Board Number: 2514

DEVELOPMENT OF MOUSE BONE MARROW MESENCHYMAL STEM CELL DISCS FOR IMPLANTATION INTO EXPERIMENTAL MOTOR CORTEX INJURIES

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It has been widely described that mesenchymal stem cells (MSC) secrete inflammatory mediators exerting an immunomodulatory function when transplanted into an injured tissue or organ. These secreted factors with immunomodulatory activity have been implicated in the promotion of tissue regeneration by stimulating proliferation, migration and differentiation of endogenous stem cells or tissue specific progenitor cells. In the brain, the main class of neuroinflamatory response molecules secreted at an injury site are the cytokines. Among these cytokines is the chemokine CXCL12, a chemoattractor for CXCR4+ cells, such as endogenous neural stem cells. Previous results from our lab showed that bone marrow-derived MSC (BM-MSC) transplanted at an injury site in the motor cortex of mice modulate the expression of the anti-inflammatory cytokines IL-4 and IL10, and the pro-inflammatory IL-6 and TNF-α. These results suggest that transplantation of MSC could have a positive effect on different CNS pathologies, such as traumatic injury, ischemia and neurodegenerative disorders. Although the presence of MSC can be beneficial to the regenerative process, in another set of experiments we observed that transplanted bone marrow-derived mononucleated cells did not remain at the lesion site and migrated towards

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the lateral ventricles, thus decreasing the period of time that they remained at the injury site. Increased permanence of transplanted cells at an injury site would likely increase their immunomodulatory effects, such as chemoattration of neuroblasts from the subventricular zone to the lesion. Based on that we aimed to develop a process to retain MSC at an injury site and use the immobilized cells as a localized source of secreted factors. In order to achieve this objective we have immobilized mouse BM-MSC imbedded in a collagen net. This system is often used in the treatment of skin burns and utilizes fibroblasts to form discs called dermal equivalents (DE). In this study we present the MSC version of DE, which we named DiMe (mesenchymal discs). DiMe was prepared with MSC obtained from adult C57BL/6-Tg-eGFP mouse femurs alone (pure DiMe) or in combination with human gingival fibroblasts (mixed DiMe). Pure DiMe was prepared by immobilizing MSC in a collagen net, and mixed DiMe was prepared by overlaying MSC on top of premade DE at different periods of time: i) during DE preparation or ii) 2, 7 or 10 days after DE was formed. After that, we analyzed the dynamics of disc formation as well as maintenance of MSC markers in the formed discs. Fixed and sectioned discs were immunostained for CD29, CD34, CD44, CD73 and nestin. We observed that the dynamics of pure and mixed DiMe contraction was similar to the dynamics of fibroblast DE contraction. Cells present in pure DiMe were positive for CD29, CD44, CD73, nestin and were negative for CD34, indicating that MSC markers were maintained after immobilization. Our results suggest that MSC immobilization might be an effective tool to be used as a form of cell based therapy for CNS pathologies.

Poster Board Number: 2516

THERAPEUTIC CAPACITY OF TYPE 2 DIABETIC BONE MARROW DERIVED MOUSE MESENCHYMAL STEM CELLS IN WOUND HEALING

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Wound healing is the result of a series of well-orchestrated events involving a sequence of inflammation, proliferation and remodeling. A systemic condition such as type 2 diabetes often disrupts the ability of the body to initiate these phases due to a loss of blood flow and defective signaling of cellular activity and growth factors. These wounds persist and often lead to amputations. Bone marrow-derived mesenchymal stem cell (MSCs) therapies are seen as a promising new avenue for repair and regenerating tissue. Native MSCs exhibit the ability to contribute to a wide variety of endogenous organ tissue repair. We have observed acceleration in closure with the engraftment of healthy non-diabetic MSCs in a slow healing type 2 diabetic mouse wound model. Recent studies have shown that grafted exogenous MSCs initiate repair but disappear long before healing is complete, suggesting significant host involvement in the repair process. This project focuses on the capacity and contribution of endogenous diabetic MSCs in the repair process. Type 2 diabetic animals (db/db) have been utilized extensively to study the pathophysiology of impaired wound healing. The characterization of stem cells, however, in a type 2 diabetic context is still largely unknown. We have derived MSCs from the db/db animal and have characterized using *in vitro* and *in vivo* assays. We evaluated proliferative capacity using BrdU, differentiation with induction media, and their therapeutic potential in-vivo by engrafting diabetic MSCs into an excisional splint wound on a diabetic and wild type mouse. Our results show that MSCs derived from diabetic animals are markedly impaired relative to their non-diabetic counterparts. The type 2 diabetic animals have fewer endogenous MSCs but this host population can be expanded over time and passage in culture. The MSCs from diabetic animals also initially exhibited efficiency problems meaning fewer proliferating cells and greater cell death compared to their non-diabetic counterparts. After multiple passages, however, a subpopulation of better performing cells demonstrated a 4-fold increase in the percentage of proliferating cells and a 3-fold increase in the number of viable cells after each passage. These higher performing cells at passage 4 were selected and used *in vivo* to test the therapeutic capacity in accelerating wound closure. The diabetic MSCs were able to accelerate closure in diabetic animals suggesting a latent ability to stimulate signaling required for healing. They were not able to accelerate closure in wild type animals, an end point that the MSCs from a non-diabetic mouse were able to achieve. Despite these *in vitro* manipulations diabetic MSCS still were unable to achieve the same levels of efficiency and therapeutic capacity as their non-diabetic counterparts suggesting an underlying impairment in the endogenous MSC population within the type 2 diabetic animals. These deficiencies in this stem cell population may have a widespread impact throughout the body and may contribute to impaired tissue maintenance, wound healing, and immune system complications.

Poster Board Number: 2518

ISOLATION OF THE STROMAL/VASCULAR FRACTION OF MURINE BONE MARROW DRAMATICALLY ENHANCES MSC YIELD, ALLOWS ISOLATION OF MARROW ENDOTHELIAL CELLS AND REVEALS A SUBPOPULATION OF STROMAL CELLS WITH LT-HSC SUPPORTING ABILITY

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Bone marrow (BM) stromal cells have been ascribed two key functions, namely as stem cells for non-hematopoietic tissues (mesenchymal stem cells, MSC) and secondly, as components of the stem cell niche that provides exogenous support for hematopoietic stem cells (HSC) in the BM. Current approaches to the study of the stromal cell system in the mouse are complicated by the low yield of clonogenic progenitors (CFU-F) reported by many investigators. Given the perivascular location of MSC in BM reported in multiple publications, we developed an alternative methodology to isolate MSC from mouse BM which is based upon maintaining the structural integrity of the stromal vascular fraction of the BM. In brief, an intact 'plug' of bone marrow is expelled from bones treated with 3 sequential rounds of enzymatic digestion to yield a single cell suspension. The recovery of CFU-F obtained by pooling the product of each digestion (1917.95+/-199) reproducibly exceeds that obtained using the standard BM flushing technique (14.32+/-1.9) by at least 2 orders of magnitude ($P<0.001$; $N = 8$) with an accompanying 196-fold enrichment of CFU-F frequency. Purified BM stromal cell populations devoid of hematopoietic contamination are readily obtained by FACS at P0 and demonstrate robust multilineage differentiation *in vitro* and *in vivo* into bone, adipose and chondrogenic progeny. A detailed immunophenotypic analysis of P0 cultures identified PDGFRab+ stromal cell subpopulations distinguished by their expression of CD105, reflecting the existence of phenotypically identical subpopulations in freshly isolated BM cell suspensions prepared by enzyme-mediated disaggregation. Analysis of serially passaged PDGFRab+/CD105+ and CD105- fractions demonstrated that both subpopulations retained their original phenotype of CD105 expression. To determine whether these stromal populations also differ in their capacity to support long-term multi-lineage reconstituting HSCs, we fractionated P0 cultures of BM stromal cells into either the PDGFRab+/CD105+ and PDGFRab+/CD105-phenotypes and tested their capacity to support LT-HSC by co-culturing each population with 10 LSKSLAM HSCs for 10 days. Following the 10 day co-culture period, the entire contents of each well was collected and transplanted into a lethally irradiated Ly5.1/Ly5.2 F1 chimeric mice along with 300,000 F1 WBM as competitor cells. Although both populations supported short-term repopulating HSC at 6 weeks, only the PDGFRab+/CD105- population was able to support long-term multilineage reconstituting HSCs at 16 weeks with all recipient mice demonstrating high level donor repopulation with an average of 61% chimerism in the peripheral blood and 51.8% donor chimerism in the bone marrow. These

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results demonstrate that the PDGFRab+/CD105- stromal cell subpopulation is distinguished by a unique capacity to support long-term reconstituting HSCs *in vitro*. In conclusion, we describe a simple and robust methodology that, for the first time, allows the simultaneous isolation of both the stromal and vascular components of mouse BM. Secondly, this technique reveals a level of stromal cell heterogeneity not apparent in previous analyses of mouse BM-derived MSC that reflects the likely complexity of stromal cell populations *in vivo*. These studies will facilitate experimental strategies designed to analyze the identity, anatomic location and functional properties of phenotypically defined subpopulations of stromal cells in mouse BM.

Poster Board Number: 2520

BUILDING A BETTER FAT CELL *IN VITRO*: TRANSCRIPTIONAL AND EPIGENETIC ANALYSIS OF STEM CELL DERIVED ADIPOCYTES VERSUS PRIMARY ADIPOCYTES

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Over the past several years it has been increasingly recognized that obesity is a strong contributing factor to a number of human diseases, including cardiovascular disease, type 2 diabetes, and cancer. In the United States, as the number of obese individuals has reached epidemic proportions, the incidence of obesity-related illness has greatly increased. In order to better understand the role obesity is playing in these disease states, it is imperative to better understand the biology of adipocytes. A well-established model of *in vitro* adipocyte function is based on the use of cultured adipose derived mesenchymal stem cells (ADMSCs). It has been shown that these adult ADMSCs can be physically transitioned from a fibroblast-like state to an adipocyte-like state using hormones and mitogens. Unfortunately, *in vitro* derived adipocytes do not accurately recapitulate many of the functional aspects of mature fat cells found *in vivo*. Unlike mature primary adipocytes, *in vitro* derived adipocytes do not readily form monocular lipid droplets, do not express high levels of the hormone Leptin, and exhibit insulin resistance. Because of these deficiencies, *in vitro* differentiated ADSMCs do not represent an ideal model for understanding fat cell biology in the context of *in vivo* functionality. It is our goal to better understand the differences between *in vitro* derived adipocytes and *in vivo* isolated primary adipocytes, with the hopes of identifying novel mechanisms which may contribute to recapitulating the "mature" adipocyte function *in vitro*. To this end we have devised a large-scale genome-wide approach to study histone H3 post-translational modifications and whole genome transcriptional profiles of both primary adipocytes and donor matched ADSMCs (undifferentiatied and differentiated). Using this technique we have identified several novel transcription factors which may play a role in fat cell development *in vivo* and contribute to achieving a more "mature" fat cell phenotype in ADMSCs derived *in vitro*.

Poster Board Number: 2522

DECREASE IN ADIPOGENESIS OF ADIPOSE-DERIVED STROMAL CELLS BY TAUROURSODEOXYCHOLIC ACID

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Human adipose-derived stromal cells (ASCs) are a heterogeneous group of multipotent progenitor cells that are possible to differentiate into various types of cells such as adipocyte, chondrocyte, osteoblast etc. Tauroursodeoxycholic acid (TUDCA) is a bile acid derivative that has been used in many countries to treat cholestatic liver disease and cholelithiasis. Recently, it has been reported that TUDCA not only has the ability to decrease endoplasmic recticulum (ER) stress, also plays a role as leptin-sensitizing agents in obese mice and human. In this study, we examined whether TUDCA affects adipogenic, chondrogenic and osteogenic differentiation of ASCs that were evaluated through RT-PCR, histological staining and so on. TUDCA significantly decreased adipogenic differentiation of ASCs, while it did not affect osteogenic and chondrogenic differentiation. These finding demonstrates TUDCA treatment on stem cells would be useful as a supplemental strategy for anti-adipogenic differentiation without changing other lineage differentiation.

Poster Board Number: 2524

MESENCHYMAL STROMAL CELLS FOR CARDIAC REPAIR

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Myocardial infarction (MI) is a life threatening disease that, despite recent advances in its prevention and treatment, causes significant mortality. Therefore, approaches based on adult stem cells represent a promising alternative to conventional therapies for MI. We followed a concept to engineer Mesenchymal Stem Cells (MSCs) which represent self renewing multipotent cells that can be isolated from multiple tissues towards cardiomyocyte progenitors. At an undifferentiated stage, MSCs have been shown to harbor capacity for cardiac repair and contributing to angiogenesis by paracrine signaling, yet so far failed to incorporate into newly forming tissue. To derive progenitors from MSCs which harbor cardiac differentiation and tissue integration potential, we exposed human MSCs to growth factors and cytokines which are known to induce the formation of early mesodermal cells in embryonic stem cells. Our data show that differentiation induction of hMSCs towards cardiomyocytes (CMs) may be possible via stimulation through Activin A or PDGF-AB, leading to expression of early cardiac or pluripotency-associated markers. Flow cytometric analysis of hMSC at 7 and 10 days post-differentiation revealed a relatively high expression of Oct4 (60% and 37% respectively), and the putative cardiac progenitor marker c-kit CD117 (14% and 18%, respectively). Strikingly, expression of cardiac transcription factor GATA4 and pluripotency marker SOX2 were also detected. Forced expression of known cardiac master regulators, Baf60c, Nkx2.5 and Tbx 5 in cells exposed to Activin A and BMP-4 further induced upregulation of early mesodermal cell markers T-brachyury (2 fold) and early cardiac marker GATA-4 (6 fold), but failed to induce transcription of mature cardiac markers such as troponin T and alpha-sarcomeric actin. When MSCs were maintained in 3D culture systems under these conditions, we observed the additional formation of large cell aggregates, and emergence of spontaneously structures beating at a frequency of 45-60/min. In summary, our data demonstrate that differentiation of MSCs via PDGF-AB or TGF-β, additional forced expression of early cardiac transcriptional regulators and 3D culture systems contribute to generating cells with a pre-cardiomyocyte phenotype.

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Poster Board Number: 2526

ANTI-INFLAMMATORY AND ANGIOGENIC EFFECTS OF ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS CULTURED IN 3D SPHEROIDS

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Mesenchymal stem cells (MSC) have been reported to possess immunomodulatory properties. We investigated whether the anti-inflammatory effects of MSC impact endothelial cells and modulate angiogenesis. In addition, we compared the immunomodulatory and angiogenic effects of MSC derived from adipose tissue that were cultured as 3D spheroids with that of MSC grown in conventional adherent 2D cultures. We measured cytokine levels after co-cultivation of 3D spheroids in a mixed lymphocyte reaction (MLR) assay. For determination of the combined effect on angiogenesis and inflammation a co-culture of MSC with endothelial cells and inflammatory cells was analyzed in a boyden chamber system regarding the effect of the MSC on angiogenesis. Previous reports suggested that culture as 3D aggregates or as spheroids can increase the therapeutic potential of the adult stem/progenitor cells. Also, MSC show induction of vasculogenesis in an inflammatory environment *in vivo*. The interaction between MSC, inflammation and angiogenesis on the cellular level is not understood. We suggest that MSC 3D spheroids derived from adipose tissue show direct anti-inflammatory effects. Furthermore, we suggest that the anti-inflammatory effects are at least partially mediated by endothelial cells. Finally, we provide data that the effect of MSC on endothelial cells is modified by the inflammatory and cellular environment.

Poster Board Number: 2528

CONTAMINATION OF MESENCHYMAL STEM-CELLS WITH FIBROBLASTS ACCELERATES NEURODEGENERATION IN AN EXPERIMENTAL MODEL OF PARKINSON'S DISEASE

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Pre-clinical studies have supported the use of mesenchymal stem cells (MSC) to treat highly prevalent neurodegenerative diseases such as Parkinson's disease (PD) but preliminary clinical trials have reported controversial results. We first observed that transplantation of human umbilical cord-derived MSC (hUC-MSC) in a rat model of PD induced by MPTP neurotoxin resulted in clinical improvement. However, surprisingly, intracerebral administration of fibroblasts as a xenotransplantation control was highly detrimental, causing significant degeneration of SN neurons and motor deficits independently of MPTP. This observation prompted us to further investigate the consequences of transplanting a heterogeneous hUC-MSC preparation, simulating contamination with human fibroblasts which is plausible since they also express typical MSC markers. Here we show for the first time, using the same experimental model and protocol, that while transplantation of hUC-MSC resulted in clinical benefit, co-transplantation with fibroblasts reverted therapeutic efficacy and caused opposite damaging effects. Engraftment and intracerebral distribution after intrastriatal administration were similar for both hUC-MSC and fibroblasts. MPTP induced significant loss of dopaminergic neurons in the substantia nigra (SN) ensuing motor deficits in sham rats. Transplantation of hUC-MSC early after MPTP injury caused a significant bilateral preservation of SN neurons and prevented later manifestation of motor deficits typically observed in PD such as hypokinesia, catalepsy, and bradykinesia. However, administration of hUC-MSC preparations deliberately contaminated with fibroblasts was not able to ameliorate the neurotoxic and behavioral effects of MPTP and further enhanced neurodegeneration in both control and MPTP-exposed rats. These findings suggest that, while hUC-MSC may induce potent neuroprotective adjustments in the brain, contamination with fibroblasts, another type of mesenchymal cell without stem cell properties, may cause opposite effects accelerating neurodegeneration. These findings might also explain discrepant results reported by different groups and indicate that purity of MSC preparations must be carefully considered in clinical trials. In addition, our pre-clinical study provides a rationale for testing MSC transplantation in early phases of PD, aiming at delaying disease progression and intensification of motor deficits.

Poster Board Number: 2530

MESENCHYMAL STROMAL CELLS FROM THE CHORIONIC VILLI OF TERM PLACENTA ARE MULTIPOTENT. HAVE EMBRYONIC FEATURES AND ARE OF MATERNAL ORIGIN

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Objective. Mesenchymal stromal cells (MSC) possess unique biological characteristics that present significant potential for regenerative therapies. MSCs were originally isolated from adult bone marrow (BM); however, adipose tissue, umbilical cord blood and matrix, and decidua of placenta are all sources of MSCs. The origin of MSCs is believed to be pericytes located in the stromal fraction of blood vessels. Thus, we hypothesized that the chorionic villi, a microvascular bed that constitutes the majority of the decidual plate, would be a rich source of placenta derived MSCs (PMSC) that may possess embryonic features and pluripotentiality. Methods. The vascular lobules of term human placenta were dissected, debrided mechanically to expose the terminal villius, then minced and digested with trypsin and collagenase. The homogenate was washed in phosphate buffered saline and cells plated on plastic with Mesocult medium for 14 days. Expanding colonies were detached and replated for purity. Cells were analyzed using fluorescent assisted cell sorting (FACS), permissive differentiation culture assays, and fluorescent *in situ* hybridization (FISH) with sex chromosome probes. Results. Placenta cells plated on plastic produced rapidly expanding colonies exhibiting MSC morphology. FACS demonstrated that pMSCs express CD 13, 29, H-CAM (CD44), 73, THY-1 (CD90), 105, 140b and did not express CDs: 14, 31, 34, 45, 133, 140a, 144 and HLA-DR, a phenotype consistent with MSCs. PMSCs express the embryonic markers Oct-4, Nanog, SSEA-3 and SSEA-4. In permissive culture assays, PMSCs were shown to differentiate into adipocytes, osteoclasts, myocytes and neurons. Using FISH analysis with Y-chromosome probes, we found that 85.6 + 1.2% PMSCs were found to be of female origin. Conclusions. PMSCs derived from the chorionic villi express the surface antigen phenotype characteristics of MSCs and uniquely possess embryonic markers. These PMSCs are capable of differentiating into multiple mesenchymal lineages demonstrating their pluripotency. PMSCs are of maternal origin; thus, there exists chimeric tolerance of fetal and endometrial vascular cells in the chorionic villi which may translate into immunotolerance with transplantation into mother or child. Most importantly, PMSCs represent a novel source of mesenchymal stem cells that may have utility in the treatment of homozygous recessive diseases such as Duchene's muscular dystrophy.

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Poster Board Number: 2532

FASL-INDUCED KILLING TARGETS MESENCHYMAL STEM CELLS DIFFERENTIATING INTO BONE MORE THAN DIFFERENTIATING ADIPOCYTES

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Mesenchymal stem cells (MSC) or multipotential stromal cells have been proposed for the regeneration of cranio-facial injuries and lesions in long bones due to their ability to form osteoblasts. However a major hindrance encountered during MSC transplantation is the initial loss of cells following implantation. This cell death is likely due, at least in part to ubiquitous non-specific inflammatory signals like those of FasL and TRAIL generated in response to the wound environment into which these cells are delivered. Our group previously found that epidermal growth factor (EGF) signaling promotes stem cell expansion while not inducing differentiation. Further, we also showed that tethering EGF (tEGF) to a biomaterial substratum protects MSC from FasL-induced cell death. These findings suggested that EGF could be used both to expand the MSC and protect them in the wound site. However, we needed to determine whether the sensitivity to inflammatory signals is altered as MSC differentiate. On subjecting MSC to differentiation, we found that MSC readily differentiated into both osteoblasts and adipocytes. However, these MSC-derived pre-osteoblasts became more susceptible to FasL-induced cell death, while differentiating adipocytes became more resistant to FasL-induced death. This coincided with receptor levels for FasL increasing in osteogenic cells and decreasing in adipogenic cells. Receptors for EGFR also increased in osteoblasts and decreased in adipocytes. tEGF did not alter the reduced cell death observed in FasL treated adipocytes. We are currently testing for survival using tEGF in differentiating osteoblasts treated with FasL. Preliminary tests with Tenascin-C a low affinity but high avidity EGFR ligand, which like tEGF also signals from the cell surface has shown to protect MSC from FasL induced cell death. Taken together, our results suggest that both MSC and differentiating osteoblasts need protective signals to survive in the implanted bone wound milieu to help regenerate bone and prevent fat formation, and that tethered EGF would serve this function.

Poster Board Number: 2534

MESENCHYMAL STEM CELLS STIMULATE THEIR OWN DEATH IN THE PRESENCE OF FASL BY PRODUCTION OF REACTIVE OXYGEN SPECIES

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Multipotential stromal cells/mesenchymal stem cells (MSC) can be differentiated into osteoblasts and chondrocytes, making these cells candidates to regenerate cranio-facial injuries and lesions in long bones. A major problem with MSC replacement therapy however is the loss of transplanted cells at the site of the graft. Studies done *in vitro* show that one of the main reasons behind this MSC death is by paracrine reactive oxygen species (ROS) signaling and effects of pro-death cytokines such as FasL and TRAIL, generated in the wound milieu. We hypothesized that MSC themselves produce ROS under cytokine challenge. To test our hypothesis, MSC were treated with FasL alone or FasL and the protein synthesis inhibitor cycloheximide. We observed ROS generation within two hours of FasL treatment that the antioxidant n-acetyl cysteine was able to curtail. Addition of epidermal growth factor (EGF), a cell survival factor was also able to protect MSC from FasL induced ROS production initially; however the protective effect waned with continued FasL exposure. Along with increased ROS, FasL induced upregulation of the uncoupling protein UCP2, the main uncoupling protein in MSC, known to reduce mitochondrially generated ROS. The increase in UCP2 seems to be a compensatory mechanism to moderate increased ROS; however the FasL treated MSC ended in apoptotic cell death. These data suggest that MSC participate in their own demise due to non-specific inflammation, holding implications for replacement therapies.

Poster Board Number: 2536

DERIVATION OF ISLET LIKE CELLS FROM MESENCHYMAL STEM CELLS USING PDX1-TRANDUCING LENTIVIRUSES

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Type1 diabetes is generally caused by insulin deficiency because of destruction of islet beta cells and differentiated mesenchymal stem cells (MSCs) can be therapeutic source for the cure of type 1 diabetes. The aim of current experiment is to explore the possibility of derivation of insulin producing cells from bone marrow mesenchymal stem cells *in vitro* by overexpression of pdx1 transcription factor. In this study rat MSCs were isolated and differentiated into adipocyte and osteocyte by differentiating medium to confirm of their multipotency. Pdx1-harboring lentiviruses were generated by transfection of three necessary plasmids (psPAX2, pMD2G, LV-105_pdx-1) into HEK293 cells. Then MSCs were transduced by pdx1-lentiviruses and pdx1-expressing cells were puromycin (2500ng/ml). The appropriate expression of exogenous pdx1 was confirmed in the level of mRNA and protein using RT-PCR and immunofluorescent analysis. The immunoflurescent showed a nuclear localization in pdx1-expressing cells. Also expression of ngn3, glucagon, insulin1, insulin2 as specific markers of β-cells was investigated by quantitative RT-PCR. These differentiated cells also expressed glucokinase (GK) and glucose transporter 2 (Glut2), indicating that the differentiated cells had the glucose-sensing ability. MSCs and pdx1-infected MSCs expressed detectable level of the mentioned genes with the exception of gapdh gene. In conclusion, MSCs as an easy and available resource can have impacted in cell based gene therapy of type1 diabetes.

Poster Board Number: 2538

EXPRESSION OF PDX1 PROMOTE DERIVATION OF BETA CELLS FROM MESENCHYMAL STEM CELLS

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The transplantation of pancreatic cells is a promising approach for treatment of type1 diabetes that generally caused by insulin deficiency because of destruction of beta cells; however, lack of suitable donors limits the application. Therefore, differentiated mesenchymal stem cells (MSCs) can be therapeutic source for the cure of type 1 diabetes. The aim of current experiment is to explore the possibility of derivation of insulin producing cells from bone marrow mesenchymal stem cells *in vitro* by overexpression of pdx1

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transcription factor. In this study rat MSCs were isolated and identified by flow cytometry that showed high level of CD29, CD90 and not detectable level of CD45 which proved the lack of hematopoietic contamination. Pdx1-harboring lentiviruses were generated. Then, MSCs were transduced by pdx1-lentiviruses and pdx1-expressing cells were selected by puromycin. The appropriate expression of exogenous pdx1 was confirmed in the level of mRNA and protein using RT-PCR and immunofluorescent analyses. In addition to ectopic expression of pdx-1 gene, differentiating medium containing nicotinamid and beta mercaptoethanol (Nico/betaME) used to efficient differentiation of rat mesenchymal stem cells into beta like cells. Then, the expression of islet specific markers was investigated by quantitative RT-PCR. The immunoflurescent showed a nuclear localization pdx1. Pdx1-expressing MSCs transcribed specific pancreatic endocrine markers such as endogenous Pdx1, Ngn3, Glucagon and insulin regardless to using Nico/betaME, but the quantitative RT-PCR showed an high increase in all markers except insulin, in Nico/betaME treated cells compared to non-treated cells. The beta like cells derived from MSCs also expressed glucokinase and glucose transporter 2 (Glut2) indicating that these cells have the glucose sensing ability. In contrast, treated MSCs didn't express P48, one of the exocrine markers; it implies that these cells have been committed to endocrine linage. In conclusion, MSCs as an easy and available resource can have impacted in cell based gene therapy of type1 diabetes.

Poster Board Number: 2540

SIGNIFICANCE OF TOPOGRAPHY AND EXTRACELLULAR MATRIX COMPONENTS IN STEM CELL DIFFERENTIATION

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When stem cells are removed from the *in vivo* stem cell niche, they can differentiate spontaneously *in vitro* but this differentiation process is inefficient, uncontrolled and often results in highly heterogeneous cell population. A crucial strategy of regenerative medicine is to understand how to control the microenvironment surrounding the cells to restore the niche equilibrium crucial for dictating stem cell fate. A typical strategy is to enrich the biochemical environment in the *in vitro* culture medium with a combination of soluble growth factors, cytokines and /or serum protein, in order to induce the stem cells to differentiate preferentially into a particular lineage. Interestingly, stem cells that were cultured on physical topographical patterns were also directed to preferential lineage, showing the importance of the physical geometrical environment in the stem cell niche. In this study, we investigated the significance of both extracellular matrixes (ECM) and topography in directing the differentiation of mesenchymal stem cells (MSCs). We hypothesize that topography is able to preferentially direct stem cell lineage through mechanotransduction while ECM components are able to synergistically direct stem cell fate. Fibronectin, vitronectin, Collagen I, Laminin and Poly-L-Lysine were coated on polydimethylsiloxane (PDMS) patterned substrates culturing MSCs on these differently coated PDMS patterned substrates using a defined medium. Immunostaining of the hMSCs demonstrated a vastly different morphology across the different ECMs, with or without the presence of physical topographical patterns. This suggests that both ECM and topographical patterns play a role in dictating the cellular morphology of MSCs. Gene expression analysis of the hMSCs however, suggests that the effect of physical topography is stronger than the constituents of the ECM coating, with a possible synergistic effect for laminin. The results provide insights to the significance of ECM and topography in directing stem cell fate.

Poster Board Number: 2542

AUTOLOGOUS CHONDROCYTES PROMOTE IN-VITRO CHONDROGENIC DIFFERENTIATION OF BOVINE MESENCHYMAL STEM CELLS

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Tissue engineering approaches to articular cartilage regeneration hold promise, yet the complexities in incorporating appropriate cell sources in bioprocessing conditions that optimize the development of tissue-engineered constructs remain a challenge. Although articular chondrocytes have been widely used in cartilage tissue engineering, several limitations have been identified, for example, the invasive surgical procedures for chondrocyte extraction and the limited ability of harvested cells to grow and proliferate. Due to multipotency and self-renewal capacity, adult mesenchymal stem cells (MSCs) have been considered a substitute for chondrocytes. However, one of the major challenges of MSC applications in tissue engineering is to precisely control their differentiation into a desired lineage. Some groups have utilized chondroinductive molecules, such as transforming growth factor-β family, to successfully induce a certain level of MSC chondrogenic differentiation *in vitro*, yet the engineered tissue fails to meet the functional criteria achieved by chondrocyte constructs. A possible explanation is that the addition of single or multiple inductive factors to the culture systems is not sufficient to induce complete MSC differentiation. Clinically, although MSC transplantation has been utilized in cartilage repair, the mechanisms that promote MSC differentiation is not fully understood. The present study aims to evaluate the feasibility of chondrocyte-MSC co-culture approaches to the regeneration of clinically relevant cartilage replacements by studying the paracrine regulation of the two populations in a simple model that mimics clinical MSC transplantation. We hypothesized that synergism between chondrocyte-generated signals can trigger and stimulate complete chondrogenic differentiation of MSCs that coexist in the system. To eliminate the interference with cell differentiation caused by medium supplements, the co-culture system was established by cultivating bovine chondrocyte pellets and autologous MSC monolayers with a serum-free, growth factor-free medium in the different compartments separated by a transmembrane film with 0.4-μm pores for 15 days. The intensity of chondrocyte-secreted signals was regulated by altering the ratios between the two populations. Harvested monolayer cells were characterized based on gene expression, matrix secretion, and morphology. Our data demonstrate that higher chondrocyte proportion is required for the monolayer cells to exhibit chondrocyte-specific gene expression profile. For example, elevated mRNA expression of type II collagen was detected in the groups where the initial chondrocyte-to-MSC ratio was at least 30. The immunofluorescent and histological staining further revealed that type II collagen and proteoglycan were highly expressed by the differentiated cells in these groups. Morphologically, the monolayer cells co-cultured with massive chondrocytes (60-fold greater in population) began forming the structure of cell clusters or aggregates after 15 days in culture. This evidence substantiates that primary chondrocytes provide essential inductive factors that are required to promote MSC chondrogenic differentiation and that the differentiated cells can mimic chondrocyte behavior and phenotype. Further development of the present model will be necessary to identify these inductive molecules and to incorporate this approach with tissue engineering strategies.

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Poster Board Number: 2544

PROTEOMIC COMPARISON OF BETA CELLS OBTAINED FROM MESENCHYMAL STEM-LIKE CELLS DERIVED FROM PANCREATIC ISLETS AND BONE MARROW MESENCHYMAL STEM CELLS

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In the present work we report a proteomic study comparing stem cells of different sources isolated by research teams of the collaborative PISTEM project of the Piemonte Region. Human islet-mesenchymal stem cells (HI-MSC) and bone marrow mesenchymal stem cells (BM-MSC) were conditioned in custom-made serum free culture media in order to induce differentiation toward pancreatic β cells. HI-MSC and BW-MSC (courtesy of Prof. Camussi, MBC, University of Turin) were phenotyped by flow cytometry for specific antigens including CD105, CD29, CD73, CD166, CD146, CD44, CD90, vimentin, and nestin. HI-MSC and BW-MSC were differentiated into β cells with a medium containing: DMEM low glucose, platelet lysate, retinoic acid, activin A, GLPI-1 (glucagon like peptide 1), EGF (epidermal growth factor), FGF (fibroblast growth factor), betacellulin, nicotinamide and glutamine. In order to demonstrate β-cell induction, indirect immunofluorescence was performed on differentiated cells with antibodies against the following proteins: PDX1 (pancreatic duodenal homeobox gene-1), insulin, and C peptide. Ultrastructural analysis revealed secretory granules. An ELISA test indicated that the cells began to secrete insulin into the culture medium after differentiation. Proteomic techniques were applied to HI-MSC and BM-MSC before conditioning to study differences in protein expression inherent to stem cells from different origins, and after conditioning to evaluate modifications of the proteomic profile after *in vitro* differentiation into pancreatic β cells. HI-MSC, BM-MSC and their derived β cells were analyzed by two-dimensional gel electrophoresis and characterized by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry analysis. All cell lines showed different proteomics patterns with few overlaps: only 11 proteins were in present in all cells. HI-MSC contained 19 unique proteins, while BM-MSC had 37. The two populations of mesenchymal stem cells expressed proteins with known functions in embryonic and postnatal growth such as STK36 hedgehog signalling pathway protein, enzymes, chaperonins and anti-oxidant proteins. β cells from different MSC origins showed a drastic decrease of the total number of proteins. Only one protein, apolipoprotein I, was induced by conditioning in β cells derived from HI-MSC and BM-MSC. A limited number of proteins were present in β cells and the original controls: PDIA1, PDIA3, GPR78, vimentin, CH60 and triphosphates isomerase. The differentiated cells maintained a proteomic profile restricted to the cell type of origin and expressed a specific set of new proteins. In conclusion the proteomic analysis give new insights in the process of differentiation: a) the differences in proteomic profile between HI-MSC and BM-MSC, b) the reduction of the number of proteins after differentiation into β cells, and c) the generation of a different repertoire of proteins upon differentiation of two MSC populations into β cells with the identical conditioning process.

PRE-CLINICAL AND CLINICAL APPLICATIONS OF MESENCHYMAL CELLS

Poster Board Number: 2546

HUMAN MESENCHYMAL STEM CELLS TRANSPLANTATION PROTECTS AGAINST SOCIAL DEFICIT EXHIBITED BY THE SUBACUTE PHENCYCLIDINE MOUSE MODEL OF SCHIZOPHRENIA

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Background: Stem cell based regenerative therapy is considered a promising cellular therapeutic approach, bringing hope for patients affected with incurable brain diseases. Mesenchymal stem cells (MSCs) represent an attractive cell source for regenerative medicine strategies for the treatment of neurodegeneration. Previous studies have shown that these cells are efficient in treating animal models of Parkinson's and Huntington's disease. Schizophrenia (Sz) is a devastating brain disease with poor prognosis. Beside a neurodevelopmental element, the pathophysiology underlying Sz involves neurodegeneration, impaired neurogenesis and alterations in neurotrophic factors availability. In the current study, we sought to explore the prospect of intracerebral MSCs transplantation for treating the social impairment endophenotype displayed by a mouse Schizophrenia model. Methods: Phencyclidine (PCP) was administered subcutaneously to C57bl mice (10mg/kg daily for 2 weeks). Adult Human Mesenchymal Stem Cell transplantation into the prefrontal cortex was conducted on the day of first PCP administration. Social preference test was conducted 10 days following the last PCP administration. Results: The results show that human MSCs transplantation into the prefrontal cortex results in a significant reduction in the impairment in social preference induced by the PCP insult, as observed by the social preference test. Importantly, no reduction of the social impairment was observed upon daily clozapine treatment. Immunohistochemical analysis of the mice brains revealed that the human cells survived in the mice brain throughout the course of the experiment (24 days). ELISA of the cortices revealed significantly higher BDNF levels in the stem cell treated group. Furthermore, Western blot analysis has shown that MSCs transplantation to the cortex prevented the significant reduction in Akt phosphorylation following the PCP treatment. Conclusion: Delivery of MSCs to distinct brain regions involved in the pathophysiology of schizophrenia is beneficial in attenuating the behavioral deficits exerted by the PCP insult. We hereby present a novel therapeutic approach for the treatment of Schizophrenia negative symptoms.

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Poster Board Number: 2548

DEVELOPMENT OF A SERUM-FREE HUMAN BONE MARROW MESENCHYMAL STEM CELL EXPANSION SYSTEM SUPPORTS SUPERIOR GROWTH PERFORMANCE AND RETENTION OF MULTIPOTENCY AND IMMUNOPHENOTYPE

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Fulfilling the clinical promise of mesenchymal stem cells (MSCs) in cell therapy and tissue engineering will require solutions to reliably expand and manufacture these primary cells. MSC expansion has traditionally employed serum supplementation of growth media, but serum presents challenges due to its lot-to-lot variation, chemically undefined nature, and potential for transmission of infectious agents. A novel serum-free MSC expansion medium, BD MOSAIC™ , yields proliferation in culture significantly exceeding that of cells in conventional serum-containing medium through multiple passages. Cells expanded in this serum-free medium maintained their multipotency in *in vitro* adipogenic, chondrogenic and osteogenic differentiation protocols as measured by lipid accumulation, glycosaminoglycan accumulation, and alkaline phosphatase induction, respectively. Serum free-expanded cells also maintained the consensus MSC immunophenotype (positive for CD73, CD90, CD105; negative for CD14, CD34, CD79a, HLA-DR). The immunomodulatory ability of serum free-expanded cells was likewise equivalent to that of serum-expanded cells in an *in vitro* T-cell proliferation assay. This chemically defined medium, developed through the application of bioinformatic and screening principles, may thus assist in the progress of MSCs from the laboratory bench to the clinic.

Poster Board Number: 2550

THERAPEUTIC APPLICATION OF HUMAN MULTIPOTENT MESENCHYMAL STROMAL CELLS FROM VARIOUS TISSUES IN ALLERGIC ASTHMA

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Multipotent mesenchymal stromal cells (MSCs) are fibroblastic cells from the bone marrow with the ability to expand in culture and differentiate into bone, cartilage and fat *in vitro*. MSCs can home to sites of tissue injury and produce soluble factors to alleviate inflammation and aid in tissue repair. Clinical experience has shown MSC administration is safe and can promote haematopoietic engraftment and control graft-versus-host disease. We have investigated alternative human tissue sources of MSCs and found that their immunosuppressive capacity is highly comparable, if not superior, to bone marrow MSCs. As systemically infused MSCs are rapidly cleared from the blood and travel to the lungs where they can exert anti-inflammatory effects, we utilised a mouse model of allergic asthma to test the efficacy of MSC treatment. Asthma is a serious and common condition, and for many sufferers there is only limited control and rarely resolution. We found that administration of MSCs decreases eosinophilic infiltration and T cell responsiveness to antigen restimulation, including proliferation, IL-5 and IL-13 production. The inhibitory effects of MSCs are crucially dependent on the number of injections and timing of treatment. Our results have clinical implications in the therapeutic application of MSCs in asthma and other inflammatory diseases of the lungs and airways.

Poster Board Number: 2554

MIGRATION CAPACITY OF HUMAN MESENCHYMAL STROMAL CELLS TOWARDS HEPATOCELLULAR CARCINOMA

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Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third cause of cancer-related death worldwide. Unfortunately, the incidence and mortality associated with HCC is increasing steadily. In the majority of patients HCC is associated with advanced fibrosis or cirrhosis. Several proinflammatory cytokines, chemokines and growth factors are produced by tumor stroma and also by cirrhotic livers. It is known that mesenchymal stromal cells (MSCs) have the ability to migrate to sites of injury or to remodeling tissues after *in vivo* administration. The aim of this work was to study the mechanisms involved in human MSCs (hMSCs) migration and anchorage to HCC in order to use them for delivery of therapeutic genes. For that purpose, *in vitro* migration was studied by modified Boyden chambers, observing that MSC displayed a high migration towards conditioned medium (CM) derived from human HCC cell lines (Hep3B, HuH7, PLC/PRF/5, HC-PT-5), from human tumor samples and also from human hepatic stellate cells (LX-2 cell line), but not to components of the stroma such as fibroblast or endothelial cells. We also observed that CM obtained from monolayer tumor cells induced higher migration in hMSCs than CM obtained from *ex vivo* tumors and also from multicellular spheroids composed by tumor cells, endothelial cells and activated hepatic stellate cells. Adhesion of hMSCs was observed at 5 min after exposure to tumor CM to fibronectin and collagen and at 30 min to endothelial cells. To further characterize the effect of HCC CM on hMSCs, invasion assays were performed observing that CM from HCC cell lines and LX-2 induced hMSC invasion to fibronectin and collagen and also increased metalloproteinase activity (MMP-2). In order to evaluate hMSC *in vivo* migration, HCC cell line HuH7 was subcutaneously inoculated in BALB/c nude mice and then CMDiI-DiR-labeled hMSCs were intravenously injected. Biodistribution of hMSCs was monitored by bioluminescence imaging using the Xenogen *In Vivo* Imaging System. Within the first hour, hMSCs located into the lungs; after 24 hours cells were observed also in the liver and spleen and around 7 days hMSCs were identified within the tumors. However, CM derived from tumors but not from spleen, liver or lungs induced hMSC migration *in vitro*. Mice were sacrificed and the presence of hMSCs was confirmed by visualization of CMDiI positive cells in the isolated organs. Our results indicate that factors produced by HCC and activated stellate cells induced hMSC migration, adhesion to extracellular matrix components and invasion capacity. Our results also demonstrate that CM obtained from monolayers cultures induced higher levels of hMSC migration in comparison with CM obtained from fresh tumors, indicating the relevance of studying hMSC migration in "a real system". A proper understanding of hMSC migratory mechanisms to the tumor sites could have important implications for effective cellular delivery of therapeutic agents in HCC therapies.

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Poster Board Number: 2556

TRANSPLANTATION OF HUMAN ADIPOSE TISSUE-DERIVED *MULTILINEAGE* PROGENITOR CELLS REDUCES SERUM CHOLESTEROL AND THE EFFECTS COULD BE AUGMENTED BY HMG-COA REDUCTASE INHIBITOR IN HYPERLIPIDEMIC WATANABE RABBITS.

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Familial hypercholesterolemia (FH) is an autosomal codominant disease characterized by high concentrations of proatherogenic lipoproteins and premature atherosclerosis secondary to low-density lipoprotein (LDL) receptor deficiency. We have supposed that human adipose tissuederived multilineage progenitor cells (hADMPCs) localized in the portal triad after transplantation via portal vein, subsequently integrated into the hepatic parenchyma and showed hepatocytic differentiation *in vivo* and lowered serum cholesterol in the WHHL rabbits, indicationg that hADMPCs-transplantation via portal vein might be a novel cell therapy strategy for the treatment of FH in the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous FH. Here we examined whether HMG-CoA reductase inhibitor pravastatin, which could correct the metabolic defects of heterogeneous FH via LDL receptor up-regulation, could augment the effect of serum cholesterol reduction onto immunosuppressed WHHL rabbit transplanted hADMPCs via portal vein. Transplantation of hADMPCs via portal vein resulted in significant reductions in total cholesterol, and the reductions maintained for 12 weeks. Then the WHHL rabbits were received HMG-CoA reductase inhibitor pravastatin i.m. or not. Four weeks after administration of HMG-CoA reductase inhibitor, the lipid profilings were examined. Total cholesterol levels and LDL-cholesterol levels of HMG-CoA reductase inhibitor treated WHHL rabbits were significantly lower than those of placebo-treated ones. On the other hand, high-density lipoprotein (HDL)-cholesterol levels of HMG-CoA reductase inhibitor treated group were higher than those of placedo-treated. To confirm the HMG-CoA reductase up-regulated LDL-receptor on hADMPCs-derived hepatocytes, we examined LDL turnover studies using 125I-labelled LDL. 125I-LDL turnover study showed that the 24 hour clearance rate of LDL was significantly higher and LDL half life was significantly shorter in the hADMPCs transplanted-WHHL rabbits treated with HMG-CoA reductase inhibitor than those of non-treated. Previously reported that hepatocytes of WHHL rabbit could not express functional LDL-receptor, and no significant effects could be observed by MHG-CoA reductase inhibitor. Our results suggested that transplantation of hADMPCs reduces serum cholesterol and the effects could be augmented by HMG-CoA reductase inhibitor in hyperlipidemic Watanabe rabbits. These results indicated the *in situ* stem cell therapy in coordination with drugs, as novel strategy for regenerative medicine.

Poster Board Number: 2558

TRANSPLANTATION OF HUMAN ADIPOSE TISSUE-DERIVED *MULTILINEAGE* PROGENITOR CELLS BUT NOT ADIPOSE TISSUE-DERIVED STROMAL STEM CELLS REDUCES SERUM CHOLESTEROL IN HYPERLIPIDEMIC WATANABE RABBITS.

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Familial hypercholesterolemia (FH) is an autosomal codominant disease characterized by high concentrations of proatherogenic lipoproteins and premature atherosclerosis secondary to low-density lipoprotein (LDL) receptor deficiency. We have supposed that human adipose tissuederived multilineage progenitor cells (hADMPCs, which were reported by Okura et al. Abstract presented at the ISSCR Meeting, Philadelphia, PA, Abstract no. 167, 2008.) localized in the portal triad after transplantation via portal vein, subsequently integrated into the hepatic parenchyma and showed hepatocytic differentiation *in vivo* and lowered serum cholesterol in the WHHL rabbits, indicationg that hADMPCs-transplantation via portal vein might be a novel cell therapy strategy for the treatment of FH in the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous FH. Here we showed that transplantation of hADMPCs but not human adipose tissue-derived stromal/stem cells (ADSCs, which were reported by Zuk et al.), could correct the metabolic defects of WHHL rabbit. To compare the effect of hADMPCs and ADSCs, human adipose tissue as excess obtained from plastic surgery was first digested in Hank's balanced salt solution containing collagenase in a shaking water bath at 37°C for 1 h. After red blood cells were excluded, mononuclear cells were collected as stromal vascular fraction. The stromal vascular fraction was divided into two aliquots and one was treated as previously reported by Zuk et al. (Mol Biol Cell 13, 4279, 2002.) to obtain hADSCs, and the other by Okura et al. (Tissue Eng Part C Methods 16,417, 2010.) to obtain hADMPCs, in brief the stromal vascular fractions were seeded, and 24h later the attached cells were treated with EDTA and the supernatant was collected and cultured as hADMPCs. Transplantation of hADMPCs via portal vein resulted in significant reductions in total cholesterol, and the reductions maintained for 12 weeks. On the other hand, the total cholesterol levels of hADSCs-transplanted group showed no significant difference to those of saline control group. To confirm transplantation of hADMPCs but not hADSCs reduces serum cholesterol in hyperlipidemic Watanabe rabbits, we examined LDL turnover studies using 125I-labelled LDL. 125I-LDL turnover study showed that the 24 hour clearance rate of LDL was significantly higher and LDL half life was significantly shorter in the hADMPCs transplanted-WHHL rabbits than those of saline control group. There was no significant difference on the 125I-LDL turnover study between hADSCs-transplanted group and saline control one. These results indicated that ransplantation of hADMPCs but not hADSCs could correct the metabolic defect of the WHHL rabbit and be a novel therapy for inherited liver diseases.

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Poster Board Number: 2560

HUMAN UMBILICAL CORD BLOOD-DERIVED MESENCHYMAL STEM CELLS PROTECT DIABETIC KIDNEY THROUGH SECRETION OF HUMORAL FACTORS

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Introduction: Excessive accumulation of extracellular matrix (ECM) and epithelial to mesenchymal transition (EMT) contribute to renal fibrosis that is associated with diabetic nephropathy, the leading cause of end-stage renal disease worldwide. Current therapy including tight control of blood glucose and pressure and inhibition of angiotensin II may delay but does not stop the development and progression of renal injury in diabetes, underscoring the need for new and more effective treatment for diabetic nephropathy. The present study examined the therapeutic mechanism as well as effect of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSC) on renal fibrosis induced by diabetes or transforming growth factor-β1 (TGF-β1). TGF-β1 is the major mediator of renal fibrosis including diabetic nephropathy. Methods: Experimental diabetes was induced by i.p. injection of streptozotocin (STZ) 50 mg/kg into 6-week-old male Sprague-Dawley rats. hUCB was harvested from deliveries with the mother's consent. MSC obtained from hUCB were administered through tail vein at the time of the induction of diabetes (2 days after the injection of STZ). To examine whether secreted humoral factors from hUCB-MSC mediate renal protection, growth-arrested and synchronized normal rat kidney tubular epithelial cells (NRK-52E) were treated with 10 ng/ml of recombinant human TGF-β1 in the presence of different concentrations of hUCB-MSC conditioned media. Results: At 4 weeks after the injection of STZ, diabetic rats showed significantly increased urinary protein excretion, renal and glomerular hypertrophy, fractional mesangial area, and renal expression of TGF-β1 and α-smooth muscle actin (α-SMA) but decreased renal E-cadherin and BMP-7 expression, confirming diabetic renal injury. Intravenously administered hUCB-MSC at dose above 1x105 cells/rat effectively prevented renal injury without significant effect on blood glucose. Engraftment of hUCB-MSC in diabetic kidneys was confirmed by CM-DiI-labeled hUCB-MSC or positive staining of human nuclei antigen (PKsc). In TGF-β1 treated NRK-52E cells, we found that hUCB-MSC conditioned media decreased mRNA and protein expression of ECM components and prevented EMT. Conclusion: The present data suggest that hUCB-MSC effectively prevented the development of ECM accumulation and EMT in diabetic kidney through secretion of humoral factors. Further investigations are required to characterize the humoral factors that are important for mediating renal protection. (Supported by HPEB-A090289 and BK21)

Poster Board Number: 2562

REGULATION OF MIGRATION CHARACTERISTICS OF HUMAN MESENCHYMAL STEM CELLS BY ENCAPSULATION IN COLLAGEN MATRIX

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Stem cell based therapies are therapeutic approaches with promising potentials in regenerative medicine. However, the success of stem cell therapy is greatly limited by the engraftment at the target tissue. In this study, we have investigated the *in vitro* migration properties and the *in vivo* engraftment rate of human mesenchymal stem cells (hMSCs) migrated through a collagen barrier. Specifically, hMSCs were subjected to a self selection process via collagen microencapsulation followed by plating onto culture dish, cells transmigrated out from the collagen barrier were characterized for their *in vitro* migration properties using transwell migration assay and *in vivo* engraftment rate using a partial hepatectomy model in NOD/SCID mice. Flow cytometry showed that the immunophenotypes of these selected hMSCs were unaltered compared with hMSCs derived from monolayer cultures. These cells also preserved their multi-potent differentiating potential and self renewing capacity, suggesting that they retain their stem cell characteristics. Upon transwell migration assay using serum free medium and chemotactic factors including SDF-1 and Fractalkine, hMSCs from both bone marrow and adipose tissue sources showed significantly higher migration efficiency than those maintained in monolayer cultures. To investigate the *in vivo* engraftment efficiency of these selected hMSCs, intravenous injection of one million hMSCs immediately after partial hepatectomy in NOD/SCID mice was performed. The remaining liver lobe was analyzed for the percentage of cells expressing human cell surface marker HLA-ABC using flow cytometry at 48 hours, 1 week and 1 month post-injection. The selected hMSCs have shown a significant increase in the engraftment efficiency comparing with those derived from monolayer cultures. These results suggest that selection through microencapsulation in collagen gel may be a possible method to enhance the engraftment efficiency of MSCs.

Poster Board Number: 2564

CHARACTERIZATION OF HUMAN MESENCHYMAL STEM CELL- NEURAL PROGENITORS (MSC-NP) DERIVED FROM PATIENTS WITH MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), with highly variable clinical course, associated with irreversible disability in a significant proportion of patients. The currently approved treatments for MS are disease-modifying agents, which only reduce the attack rate and delay progression in some patients, and no treatment is available to stop or reverse the progression of the disease. Mesenchymal stem cell (MSC)-based therapies offer an autologous source of stem cells with potential for treatment of progressive forms of MS. We have investigated bone marrow mesenchymal stem cell-derived neural progenitors (MSC-NP) as a subpopulation of MSCs which are optimized for CNS applications due to their neural progenitor, immunoregulatory and trophic properties, as well as a reduced capacity for mesodermal differentiation. Preclinical testing has demonstrated efficacy of MSC-NPs in experimental animal models of MS. The aim of this study was to characterize MSC-NPs obtained from bone marrow aspirates from a panel of healthy and MS donors. MSCs were isolated and expanded from three healthy donors, one spinal cord atrophy patient, and ten patients with secondary progressive form of MS (SPMS). To obtain MSC-NPs, MSCs were cultured for 3 weeks in a serum-free neural progenitor maintenance media. MSC-NPs consist of a subpopulation of MSCs and exhibit characteristic neurosphere morphology. Gene expression changes in MSC-NPs compared to the MSCs from which they were derived was determined by quantitative real-time PCR. We analyzed a panel of genes consisting of stem cell markers, markers of neural or mesenchymal lineage potential, and genes potentially involved in the immunoregulatory and trophic properties of MSCs and MSC-NPs. Neural lineage markers Nestin (neural stem cell marker), medium neurofilament (neuronal marker), GFAP (glial and neural stem cell marker) were all upregulated 5 to 10 fold in MSC-NPs compared to MSCs, confirming the neural lineage potential of MSC-NPs. In addition, C-X-C chemokine receptor

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type 4 (CXCR4, stem cell marker) was upregulated approximately 100 fold. Conversely, MSC-NPs expressed decreased mRNA levels of MSC markers CD90 and smooth muscle isoform of alpha-actin (SMA) with a 0.5 to 0.1 fold change compared to MSCs. Time course experiments demonstrated that gene expression changes were maximal after one week of MSC-NP induction and sustained for up to 3 weeks. In addition to neural markers, a number of immunoregulatory markers such as IDO1, TGF-β, TLR2, TLR3, TLR4, IL-10, CXCL-10, and IL-6 and potential trophic mediators such as GDNF, HGF, IGF, IL-11, VEGF-A, CNTF, and BDNF were also upregulated in MSC-NPs compared to MSCs. Changes in gene expression were confirmed by protein analysis. There were no differences in gene expression changes in MSC-NPs between control or MS patients. The observed gene expression changes in MSC-NPs give insight into the potential therapeutic mechanisms by which MSC-NPs may influence CNS repair *in vivo*. Furthermore, these data contribute to the preclinical characterization and identification of MSC-NPs for future therapeutic use in MS.

Poster Board Number: 2566

VEGF PRODUCTION IN RESPONSE TO TNFA IN HUMAN MSCS GROWN IN AMBIENT OXYGEN AND HYPOXIA

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VEGF production in response to TNFa in human MSCs grown in ambient oxygen and hypoxia. Abstract: Human MSCs upregulate production/secretion of paracrine factors with appropriate stimuli, such as when challenged with TNFa, LPS, hypoxia. We determined the secretion of VEGF in response to increasing amounts of TNFa in MSC grown in either ambient oxygen or hypoxic conditions. Our results suggest that in hypoxic cells VEGF production continues to increase with higher TNFa levels in contrast to normoxic cells which reach the maximum VEGF secretion at lower concentrations of TNFa. Background: Human mesenchymal stem cells are promising therapeutic agents for diseases due to their ability to home towards and repair injured tissues, at least in part due to their release of paracrine factors, such as VEGF, FGF2, IGF-1 and HGF. *In vitro* stimulation by TNFa and TGFa results in increased production of VEGF, as do other stimuli such as LPS and acute hypoxia. In this paper we compared production of VEGF in response to TNFa by MSC grown in ambient and low O2 conditions. Methods: Human MSCs were purchased from Lonza and expanded in tissue culture in DMEM containing 15% bovine growth serum and 10 ng/ml bFGF at 370C, 5% CO2 either in ambient oxygen (normoxic) or low (5%) oxygen. MSC at passage 4 were seeded on tissue culture dishes and incubated in appropriate conditions till they reach about 70% confluence. DMEM without bFGF but with or without TNFa was added to the plates and collected 24 hrs later for determination of secreted VEGF by ELISA. Results: When cells were stimulated with various concentrations of TNFa, there was an increase in VEGF production. The increase reached plateau in cells grown in ambient oxygen. Interestingly, cells grown in hypoxic environment showed a continued increase in the levels of VEGF produced. Legend: Controls are cells grown in ambient and 5% oxygen without TNFa (RC & LC); TNFa stimulation at 25 ngs/ml (RT25 and LT25), 50 ngs/ml (RT50 & LT50) and 100 ngs/ml (RT100 and LT100) Conclusion: MSC grown in low O2, in contrast to normoxic cells, showed continuously increased production of VEGF at higher TNFa concentrations. This finding may have important implications for therapeutic efficacy of MSC.

Poster Board Number: 2568

NEW AND EFFICIENCT METHOD FOR SCALING UP HUMAN MESENCHYMAL STEM CELLS

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The use of single layer T-flasks can prove to be time consuming, cumbersome and labor intensive for cell culture applications requiring large numbers of cells. Yet, caution needs to be exercised while scaling up cells using multi-layer vessels as potential differences in culture environment within vessels can lead to subtle changes that can affect yield, homogeneity and performance of cell populations. In the current study we compared the growth of human mesenchymal stem cells (hMSCs) in single layer BD T-175 flasks with those grown in BD Falcon Multi-flasks (3-layer). hMSCs were grown in media with and without serum. For serum free culture, vessels were pre-coated with attachment factors. Cells could be thawed directly into the multi-layer vessels obviating the need for step-wise scale up. Overall, cell yield from both single and multi-layer vessels were comparable when normalized for cells/cm2. Cell proliferation profile of hMSC populations were uniform following scale-up in multi-flasks and comparable to those grown in single layer T-175 flasks. Data regarding comparability of cell multipotency and immunophenotype maintenance between cells cultured in single and multi-layered vessels will be presented. In addition to the hMSCs, diverse established and primary cell lines have also been cultured in the BD Multi-layer vessels with equivalent performance to T-175 flasks. This study demonstrates a new and efficient way to scale-up stem cells without the need for re-optimizing existing cell culture conditions or compromising the quality, performance and homogeneity of cell populations.

Poster Board Number: 2570

GENES ASSOCIATED WITH ISCHEMIA-INDUCED VEGF SECRETION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS

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Secretion of vascular endothelial growth factor (VEGF) is known as one of the important mechanisms of ischemic damage repair mediated by human mesenchymal stem cells (hMSCs). Therefore, we attempted to identify candidate genes that can serve as markers to predict VEGF secretional level of hMSCs under ischemic condition. Six lots of bone marrow-derived hMSCs were cultured under normal or ischemic condition. The cell culture media were collected 24hrs after the exposure to the ischemic stress, and their VEGF levels were measured by ELISA. Then, we analyzed the correlation between their gene expression profiles under the normal condition (Affymetrix HG-U133Plus2.0) and their VEGF secretion after the exposure to the ischemic stress. Seventeen genes showed positive correlations in all combinations of pre-ischemic gene expression (passage 7 or 9) and post-ischemic VEGF level (amount or %change) ($p<0.01$, Spearman's rank correlation coefficient). Furthermore, RNAi against 2 of the 17 genes reduced VEGF secretion in response to the ischemic stress ($p<0.05$). These findings indicate that the 17 genes may be useful to assess quality and efficacy of hMSCs

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under ischemic environment. In addition, it is suggested that the 2 genes, knockdown of which decreased the VEGF response, are functionally coupled to the ischemia-induced VEGF secretion of hMSCs.

Poster Board Number: 2572

INTRACEREBRAL TRANSPLANTATION OF HUMAN PLACENTA-DERIVED MESENCHYMAL STEM CELLS FOR THE NEUROREGENERATION IN A RAT PERINATAL BRAIN INJURY MODEL

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Objective: Peripartal brain injury in the premature infant leads to a large spectrum of clinical problems. There is no established therapy available. Stem cell transplantation has been proposed as a therapy for neurodegenerative diseases. The aim of this study is to assess the therapeutic potential of human stem cell treatment in an animal model of perinatal brain damage. Methods: Perinatal brain injury was induced in newborn (postnatal days 2-7) Wistar rats by the administration of LPS (0.1 mg/kg BW, i.p.), followed by the ligation of the left carotid artery and hypoxia (8% O2, 80 min). Injured and sham-treated animals were transplanted 24h, 1 week or 4 week post injury. Newborn anesthetized rats were fixed in a stereotaxic frame (Kopf) and mesenchymal stem cells (MSC) derived from human placenta (chorion) or umbilical cord Wharton's jelly (250'000 cells in 5 µl) were injected into the left lateral ventricle using a 32-gauge needle at a very low speed (Lab Animal Studies Injector, Hamilton). The animals were sacrificed 3-4h, 1 week, 2 and 4 weeks after transplantation. In brain sections, donor human MSC were detected by immunohistochemistry using a mouse anti-human HLA Class 1 ABC antibody. Co-labeling using antibodies against neurons, astrocytes and oligodendrocytes are used to detect donor cell differentiation into the neural lineages Results: Single donor-derived cells detected in the brains of healthy rats shortly (1-2h) after transplantation showed an unaltered morphology. After 4h, donor cells had started migrating throughout the ventricular system. A few cells had already migrated into the parenchyma. Two weeks after transplantation, 84% of the uninjured pups had survived the transplantation procedure. The first 24h after transplantation were crucial for the survival. Single cells were found in the solid tissue around the ventricles. Conclusion: Human stem cells were successfully delivered into the lateral ventricle of neonatal rat brains. Donor cells were detected in the hosts' brains up to four weeks after transplantation. Longer term studies will be done to analyze the proliferation, the long-term survival and engraftment of the donor cells. The co-expression of lineage-specific proteins and human-specific markers will give information about the fate of the transplanted human cells in the brains of both injured and control rats. As a proof of principle, stem cell-treated and non-treated rats will be subjected to behavioral tests to assess the improvement of motor and memory deficits.

Poster Board Number: 2574

NEURAL DIFFERENTIATION POTENTIAL OF HUMAN UMBILICAL CORD WHARTONS JELLY-DERIVED CELLS

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OBJECTIVE: Perinatal brain damage accounts for the major part of the clinical problems in surviving premature infants. A considerable therapeutic potential has been ascribed to mesenchymal stem cells. The umbilical cord connective tissue (Wharton's jelly) represents a promising source of mesenchymal stem cells. Thus, the aim of this study is to characterize the phenotype of human Wharton's jelly-derived cells and to assess their neural differentiation potential. STUDY DESIGN: Wharton's jelly cells from umbilical cord tissues of term (gestational age ≥ 37 weeks) and pre-term (gestational age: 27 - 36 weeks) pregnancies were evaluated. The expression of the membrane markers for mesenchymal stem cells defined by the International Society for Cellular Therapy (ISCT) was measured by flow cytometry. Accordingly, mesenchymal stem cells have to be ≥ 95% positive for CD105, CD90 and CD73, but negative for CD45, CD34, CD19, CD14 and HLA-DR. Adaptations of previously published multistep protocols were used to produce neural progenitors and, subsequently, induce terminal neural differentiation. Analysis of neural differentiation markers was performed by real-time PCR, flow cytometry and immunocytochemistry. RESULTS: Isolated umbilical cord Wharton's jelly cells were plastic adherent and highly positive for CD105, CD73, CD90, but negative for CD45, CD34, CD14, CD19 and HLA-DR, independent of gestational age. A subset of Wharton's jelly cells displayed the neural progenitor cell marker nestin, whose protein and gene expression was increasing during neurogenic pre-induction into neural progenitor cells, the so-called neurospheres. In addition, mRNA levels of the neural progenitor cell markers PAX6, musashi-1 and nanog were elevated in neurospheres relative to undifferentiated Wharton's jelly-derived cells. Conclusion: Wharton's jelly cells meet the criteria for the phenotypic characterization of mesenchymal stem cells. The up-regulated expression of neural progenitor cell markers in neurospheres compared to undifferentiated cells strongly indicates that neural precursor cells can be obtained from Wharton's jelly-derived mesenchymal stem cells. Terminal differentiation into the neural subtypes is currently tested.

Poster Board Number: 2576

DISTRIBUTION OF TRANSPLANTED HUMAN MESENCHYMAL STEM CELLS DURING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Mesenchymal stem cells (MSCs) are promising vehicles for the treatment of neurodegenerative diseases such as multiple sclerosis (MS). This is in part due to their broad immunomodulatory properties and their reported ability to home to sites of tissue inflammation and injury. We have previously demonstrated that human mesenchymal stem cells (MSCs) derived from the bone marrow (BM-MSCs), adipose tissue (Ad-MSCs) or umbilical cord Wharton's jelly (UC-MSCs) were capable of suppressing the *in vitro* prolif-

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eration of lymphocytes stimulated by either non-specific or specific mitogens, even in the absence of cell-to-cell contact. Transplantation of all three types of MSCs suppressed the clinical and pathological signs of disease in mice with either chronic progressive (CP) or relapsing-remitting (RR) forms of the MS-like disease termed experimental autoimmune encephalomyelitis (EAE). Given these results, mechanisms focusing on the fate and homing of systemically administered MSC to the central nervous system (CNS) or secondary peripheral lymphoid organs have not been clearly defined. In the current investigation we analysed the expression of molecules involved in cellular trafficking from the blood into tissues and tracked gene-modified MSCs in normal and immunodeficient mice. We have further analysed the post-transplant fate of MSCs in mice with CP- and RR-EAE. FACS analysis of integrins, selectins, members of the immunoglobulin superfamily and tetraspanins, which are involved in cellular adhesion, revealed that all three MSC groups expressed a broad range of cell surface molecules including CD29, CD44, CD49b, CD49c, CD49e, CD63, CD81, CD106, CD151 and CD166. RT-PCR analysis also confirmed the expression of the chemokine receptors CCR6, CCR7, CCR11, CXCR3, CXCR6 and CXCR7. Interestingly, Ad-MSCs and UC-MSC expressed a number of common markers such as CD9, CD49a, CD51, CD54, CD61, CCR9, CCR10 and CXCR2, whilst BM-MSCs did not. Significantly, exposure to INFg did not result in the upregulation of these chemokine receptors. *In vivo* bioluminescent tracking of transplanted MSCs engineered to over express luciferase in immunocompetent Balb/c mice or immunocompromised NOD/SCID mice revealed that after intravenous infusion, most cells were trapped as emboli in the lung that persisted for approximately 3 to 4 weeks. We next examined the distribution of human BM-MSCs expressing eGFP in CP-EAE. In this model, C57Bl/6 mice were immunized with recombinant myelin oligodendrocyte glycoprotein (rMOG) and twelve days later, during the onset of disease signs, BM-MSCs were infused. Lymph nodes, spleen, CNS, heart, liver, gut and lung tissues were excised and examined for the presence of MSCs. PCR analysis of extracted DNA from these tissues using eGFP-specific primers revealed that donor-derived cells were only transiently located in the lung or spleen. On-going studies characterizing the distribution of gene-modified MSCs into NOD/Lt mice immunized with rMOG, which manifest a RR-form of EAE, are currently underway. In summary, these results suggest that a low number of persisting MSCs, which were below the detection of methods used in this study, can elicit long-lasting anti-inflammatory effects were they limit the number of lesions, reduce axonal loss and preserve myelin structure. Future mechanistic studies will investigate the gene expression patterns of transplanted MSCs, particularly with cells that have lodged in the pulmonary vasculature.

Poster Board Number: 2580

EFFECTS OF SYSTEMIC TRANSPLANTATION OF AUTOLOGOUS MESENCHYMAL STEM CELLS ON THE REGENERATION OF RAT NERVOUS TISSUE AFTER CLOSED CRANIOCEREBRAL TRAUMA

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We studied effects of transplanted mesenchymal stem cells (MSC) from Wistar rat bone marrow on the functional morphology of neurons and reparative processes in the nervous tissue of rats after closed craniocerebral trauma. The brain trauma was modeled by a free falling weight (impact area 0.5 cm2). Survived animals (about 50 from 100) were divided into the comparison groups. Group 1 included rats with the injury without treatment. Group 2 included rats with the injury receiving basic therapy (antiedematous, antihypoxic, and anti-inflammatory drugs). Group 3 consisted of rats who received a single i/v injection of 2 x 106 MSC 24 h after the injury. Group 4 involved rats who received a combined treatment: basic therapy and the MSC injection. The control group consisted of sham-injured rats. The rats were euthanized in 1 h, 1 day, 3 days, or 14 days after the injury. The brains were isolated, fixed and then cut into two sagittal blocks or 4 frontal fragments at the level of the rostral compartment of the lateral ventricles, hippocampus, midbrain, and cerebellum. Sections were stained using Nissl method. PCNA immunostaining was used for the cell proliferation analysis. Histotopographic mapping of the damaged areas and precise determination of the levels of coronary sections were carried out using a stereotaxic atlas of the rat brain. We have found that closed craniocerebral trauma causes diffuse damage of the rat brain. The primary injury is associated with the vascular disorders and traumatic edema peaking on the 1st day. The edema leads to ischemic damage of the cortical and brain stem neurons. The reactive and compensatory-regeneratory mechanisms along with cell proliferation in the zones of neurogenesis are triggered during the earliest periods after the injury. For Group 2, the effect on the proliferative activity of cells in vascular plexuses, microglia, and particularly, ependymal and subepedymal layers appeared only on the 3rd day after the trauma. After 2 weeks, despite recovery of the neuron morphology, there were still some local foci of cell proliferation and elevated activity of neurogenesis zones in the cortex, corpus callosum, and diencephalons. The therapeutic effect in Group 3 was observed visually on the 2nd day after intravenous injection of MSC. The neuroprotective effects of MSC on the diffuse brain injury included not only stimulation of general reparative processes during the regeneration phase (rapid restoration of Nissl substance in neurons, acceleration of gliogenesis and angiogenesis), but also, presumably, activation of the germinative zones of neurogenesis. Systemic transplantation of MSC activated other cells additionally expressing growth factors in the brain such as glial cells expressing neurotrophic factors to promote the neurons' survival and recovery. Thus, systemic injection of MSC in a syngeneic organism produced proliferotropic, angiogenic and presumably neurotrophic effects. In Group 4, the activating effect of MSC was augmented by the basic therapy. We found the combined treatment including metabolic support and i/v MSC injection to create prerequisites for activation of the mechanisms of compensatory adaptive processes during the reparative regeneration period and for improvement of nervous tissue trophics, essential for the restoration of the functional morphology of damaged neurons. These morphological results are consistent with the additional results on the dynamics of psychophysiological tests of rats from the same groups.

Poster Board Number: 2582

ENGRAFTED DENTAL PULP STEM CELLS PROMOTED FUNCTIONAL RECOVERY OF COMPLETELY TRANSECTED RAT SPINAL CORD

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Adult spinal cord injury (SCI) often leads to persistent functional deficits due to insufficient neuropoiesis and limited axonal regeneration of injured CNS: no effective treatment to cure the SCI has been established. We report here that dental pulp stem cells from human exfoliated deciduous (SHEDs) and adult permanent teeth (DPSCs), when transplanted into the acute phase of completely transected rat spinal cord, significantly recovered hind-limb locomotors functions. SHEDs strongly suppressed apoptosis of neurons, astrocytes and oligodendrocytes, and preserved neuronal filaments and myelin sheaths after complete transection. SHEDs regenerated both corticospinal tract (CST) and raphe spinal serotonergic (5-HT) axons beyond the epicenter. Interestingly, before transplantation, more than ninety-five percent of both SHEDs and DPSCs uniquely co-expressed variety neuronal markers being expressed by neural stem cells, neuroblasts, early neurons,

astrocytes and oligodendrocyte progenitors. We found that, after eight weeks from transplantation, SHEDs specifically differentiated into the mature oligodendrocytes expressing myelin basic protein and APC; concomitantly abolished expressions of neurofilaments and astrocytes. Taken together our data demonstrate significant benefits of SHEDs and DPSCs-based regeneration therapy for treatment of SCI. In the acute phase, factors secreted from SHEDs would protect injured spinal cord from severe neural damages in none-cell autonomous manner; subsequently, specifically self-differentiated SHEDs into the oligodendorocytes contributed functional recovery by promoting re-myelination of damaged axons in cell autonomous manner. Thus both none-cell and cell autonomous neuro-regenerative activates of tooth derive stem cells play important role in functional recovery of SCI.

Poster Board Number: 2584

HUMAN IMMATURE DENTAL PULP STEM CELLS (HIDPSC) IN CHRONIC DOG SPINAL CORD INJURY - PRELIMINARY RESULTS

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The regenerative process of a injured area of the central nervous system (CNS), particularly the spinal cord, has some limiting factors, such as the limited capacity of the CNS to restore damaged cells. Despite several therapeutic attempts have been made the results remain unsatisfactory, even using human models. This study aims to evaluate the clinical outcomes of transplanted human immature dental pulp stem cells (h-IDPSC) in dogs with different degrees of chronic spinal cord injury, unresponsive to conservative treatments. Magnetic resonance imaging (MRI) and the Olby et al. (2001) behavioral scale were used to analyze and classify each animal response to treatment. In this experiment there were used five animals. Those whose chronicity of the injury prevented the surgical treatment, compounded the experimental group. All animals included in the research protocol were evaluated by preoperative MRI. The findings of MRI from the first clinical case indicated the presence of myelopathy with mild extradural cranial hydromyelia caused by ventral disc material in the right side of T12-13. A corpectomy associated with pediculectomy was performed under general anesthesia, and 1 x 106 h-IDPSC were injected by intramedular route. After this procedure, the animal was submitted to veterinary physiotherapy for muscular tonicity and proprioceptive reflexes increase. It was possible to observe a gradual and progressive improvement in the patient behavior, measured with the method proposed by Olby (2001). After 15 days of surgery, it could be classified with a score around 4 or 5, and this rank reached 7 after 30 days. Postoperative MRI showed the resolution of the initial hydromyelia process, leading to an apparent improvement of pre-existing condition. These findings lead us to believe the h-IDPSC helped in the process of regeneration of the spinal cord.

Poster Board Number: 2586

CELL THERAPY IN ADULT DOGS WITH CHRONIC SPINAL CORD INJURY USING BONE MARROW STEM CELLS DERIVED FROM DOG FETUSES - PRELIMINARY RESULTS

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Spinal cord injury is one of the most devastating damage, especially when installed abruptly, with aspects physicals and psychologicals, confronting the medicine with multiple challenges. The regenerative process of the central nervous system (CNS), and particularly in spinal cord, has some limiting factors, such as the limited capacity of the CNS to restore the damaged cells. Moreover, considering all the problems and difficulty in finding satisfactory treatment, the cell therapy emerges as a possible tool for the treatment of such injuries. Our group proposes fetuses bone marrow stem cell as a promising source of cells to treat spinal cord injury. So, in our work we use magnetic resonance imaging for effective diagnosis of spinal cord injury and implant bone marrow stem cells derived from fetal dogs in the lesion focus through surgery. The dogs treated with the cells were undergoing physical therapy, and their performance was recorded and evaluated by a veterinary physiotherapist, using also a behavioral test (Test of Olby). Special attention was done to look for evolution in the march and in urodynamic function. Until the present moment two animals were submitted to the treatment and both of them showed an improvement in the Olby score and in the urodynamic function. These findings lead us to believe that the stem cells from fetuses bone marrow helped in the process of regeneration in the spinal cord.

Poster Board Number: 2588

ANTITUMOR EFFECTS OF GM-CSF OVEREXPRESSING CANINE ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS IN A MOUSE MELANOMA MODEL

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Cell-based therapy is a promising strategy to treat cancer. Among cell types that can be used for this purpose, mesenchymal stem cells (MSCs) appear to hold great advantage for reasons including easier propagation in culture, possible genetic modification to express therapeutic proteins and homing to sites of cancer growth upon *in vivo*. Accordingly, the primary objective of the present study is to investigate the preventive and therapeutic potential of canine AT-MSCs against B16F10 melanoma in a mouse model. Secondary, this study examines the preventive antitumor effects of GM-CSF overexpressing cAT-MSCs in experimental melanoma model. C57BL/6 mice (six to eight-week-old, female) from the Charles River Laboratories (Wilmington, MD) were immunized with 2 x 105 unengineered cAT-MSCs (antigen-pulsed/unpulsed) or GM-CSF-secreting cAT-MSCs (antigen-pulsed/unpulsed) on day -13, -10, -7, before the challenge with 2 x 105 tumor cells on day 0. In all experimental design, mice were monitored for the formation of palpable tumors twice weekly and tumors were measured three times a week. The present study shows that cAT-MSCs have the potential to inhibit the proliferation of B16F10 melanoma *in vitro* and to attenuate rapidly growing tumor cells in a mouse melanoma model. In addition, cAT-MSCs were able to perform cross-presentation of B16 antigen and inhibit the growth of the B16 melanoma cells *in vivo*. Mice injected with unpulsed cAT-MSCs developed tumors that reach a mean tumor volume of 1552 mm3. In contrast, a mean tumor volume of mice injected with the B16 antigen-pulsed cAT-MSCs reached only 566 mm3 ($P < 0.05$) at 22-day time point. Mice injected with the unpulse mGM-CSF overexpressing cAT-MSCs develop tumors that reach a mean tumor volume of 2801 mm3 at day 22 after tumor cell injection, as determined with 4 mice. Mice injected with the B16 antigen-pulsed mGM-CSF overexpressing cAT-MSCs develop tumors that reach a mean volume of 1511 mm3 at this 22-day time point. GM-CSF has been found to be more potent in generating a long-lived, specific, systemic antitumor response than any other single cytokine examined if GM-CSF production is above 35 ng per 106 cells per 24 hours. However, the GM-CSF dose released by cAT-MSCs in this study (1969.8 pg per 106 cells per 24 h) was insufficient to have anti-tumor activity that appears to be related to

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stimulation of the immune system. In conclusion, the present study indicates that AT-MSCs remarkably attenuate the growth of B16F10 melanoma cells *in vivo* and *in vitro*. Also AT-MSCs can cross-present B16F10 antigen induce an effective immune response, a property that could be a useful tool for cancer cytotherapy.

Poster Board Number: 2590

NEURONAL INDUCTION OF WHARTON'S JELLY-DERIVED MESENCHYMAL STROMAL CELLS USING ROCK INHIBITOR IMPROVES THERAPEUTIC EFFECT IN INTRACEREBRAL HEMORRHAGE STROKE MODEL

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Mesenchymal stromal cells (MSCs) are known to differentiate into mesodermal lineage cells and neuronal cells in specific conditions. Rho-kinase (ROCK) inhibitors have therapeutic effects in animal models of CNS disorders and co-treaement of ROCK inhibitor and CoCl2 has been reported to stimulate neuronal differentiation of murine bone marrow MSCs. Here, we isolated human MSCs from Wharton's jelly in umbilical cord (WJ-MSC) and demonstrated neuronal differentiation by combination treatment of ROCK inhibitor and CoCl2 and promoted therapeutic effect of ROCK inhibitor-primed MSCs in brain ischemia The combination treatment of ROCK inhibitor and CoCl2 in WJ-MSCs in a special induction medium provoked morphological changes into neuron-like cells and increased expression of neuronal markers such as Tuj-1, NSE, and NF-H. Accordingly, ROCK knockdown by ROCKI, II specific siRNAs also resulted in induced axonal process and upregulated MAP2 expression in WJ-MSCs. In order to confirm the therapeutic effect of neuronal induction of WJ-MSCs by ROCK inhibitor in neuro-degenerative disease, induced- or none induced WJ-MSCs cultured in the presence or absence of ROCK inhibitor were transplanted into rat intracerebral hemorrhage (ICH) model. Behavior of the transplanted animals was evaluated for period up to 8 weeks using modified Rotarod test and limb placement test. The induced WJ-MSC transplanted group showed markedly improved functional performance compared with the PBS group after 3 weeks and compared with the none-induced WJ-MSC transplanted group after 7 weeks on Rotarod test. In addition, the engrafted WJ-MSCs were detected by immunostaining with anti-human mitochondria specific antibody and anti- MAP2, NF-H or GFAP antibody. In the induced WJ-MSC group, 52.29% of the grafted cells were MAP2 positive in contrast to 3.17% of the none-induced WJ-MSC group. Interestingly, the induced WJ-MSC represented strong expression of GDNF in the tissue of ICH region. Accordingly, WJ-MSCs secreted higher level of GDNF upon treatment of ROCK inhibitor *in vitro*. Altogether, these results suggest that neuronal induction of WJ-MSCs by ROCK inhibition is a possible application for cell replacement therapy of ICH and other neurological disorder diseases. NRF fund from the MEST (2009-0079656)

Poster Board Number: 2592

PRECLINICAL CELLULAR THERAPEUTIC SAFETY STUDY DESIGN CONSIDERATIONS

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Safety evaluations of cell-based therapies present unique challenges in preclinical study design and execution. Preclinical studies must be specifically designed for each cell product and indication to provide the developer valuable information for assessment of potential clinical risks and address the regulatory hurdles to be overcome to bring the therapy to market. In these studies, a well characterized cellular product must be assessed for cell survival, differentiation (anticipated or unanticipated), distribution, and neoplastic potential in an appropriate preclinical model. These design considerations include selection of the appropriate test system (including relevant surgical animal models), extent of cell characterization both before and after administration, study duration, critical toxicological endpoints, and required cell detection criteria to evaluate deposition, distribution and cell fate. These properly incorporated study design components, implemented by GLP-compliant laboratories with the required cell and animal model capabilities and reported by experienced preclinical scientists, constitute a complete experimental program. These comprehensive preclinical evaluations, conducted with respect to the most current understanding of the regulatory climate in which this work is evaluated, are essential for success in meeting the safety and regulatory expectations.

Poster Board Number: 2594

CELL THERAPIES FOR INFLAMMATORY DISEASES: BIOLOGICAL BASIS FOR NEW CLINICAL PROTOCOLS

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Chronic inflammation is involved in the pathogenesis of several human diseases, such as cancer, atherosclerosis, allergy, inflammatory bowel diseases and autoimmune diseases. Several anti-inflammatory pharmacological drugs are currently available but with limited efficacy and deleterious long-term consequences. Long lasting tolerance induction may represent a safe and effective resolution for these diseases and scientists have become interested in therapies based on cells with immunomodulatory activity. It has been widely demonstrated that Mesenchymal Stem Cells (MSCs) exert potent immunosuppressive effect both *in vitro* and *in vivo*, although the results of clinical trials are still disappointing. This can be due to several reasons including the fact that MSCs display a low ability to home to inflamed tissue. In addition, the biological mechanisms responsible for MSC-induced immunomodulation are still not clear. Our project aims at a deeper understanding of the MSC biology, tolerogenic properties and homing. In particular we have transplanted alginate-encapsulated MSCs in order to determine whether their therapeutic effect is dependent on migration capacity and cell to cell contacts. Using TCR transgenic mice, we have found that encapsulated MSCs hamper recruitment of leukocytes and dendritic cells into inflamed lymph nodes and antigen-specific CD4+ T cell proliferation *in vivo*. This immunomodulation is thus independent of MSC homing to specific tissues and likely due to a direct effect on endothelial activation and permeability.
Furthermore, we found that the administration of encapsulated MSCs was more effective than their intra venous administration, with the advantage of being safer and removable. These results suggest that the efficacy of MSC administration in clinical protocols aimed at treating inflammatory diseases can be greatly improved.

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Poster Board Number: 2596

SAFETY AND EFFECTS OF ALLOGENIC BONE MARROW MESENCHYMAL STEM CELL TRANSPLANTATION IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Systemic Lupus erythematosus (SLE) is a multisystem autoimmune disease. Despite the advances in immunosuppressive medical therapies, SLE remains fatal in some patients. In this case report we used allogenic bone marrow mesenchymal cells (BM-MSC´s) based on studies that shows that MSCs from some patients with lupus may have abnormalities in phenotype, proliferation, differentiation and senescence. In addition the cells lose their immunomodulatory functions. To evaluate the feasibility, safety, and immunological effects of BM-MSC´s, we compare the growth characteristics, cell membrane markers (CD34-, CD105 +, CD144 +, CD90 +, HLADR-), cell differentiation to adipogenic, osteoblastic and chondrogenic lineages, and karyotype in one patient and in one healthy donor. One female, 28 years old fulfilling the 2002 American College of Rheumatology (ACR) criteria for SLE in mild stage was infused with allogenic BM-MSC´s. We evaluated the suppressive effect of these cells *in vitro* and changes in CD3+, CD4+, CD8+, CD25 +, FoxP3 + in regulatory T cells from the patient and proinflammatory cytokines before and after treatment. Allogenic BM-MSC´s transplantation is a safety treatment with no adverse effects in the patient. These cells are capable of reverse multiorgan dysfunction resulting in amelioration of disease activity and stabilization of proinflamatory cytokines according to the clinical trials conducted by Dr. Sun, with a decrease in the proliferative responses of lymphocytes. Our case report point out in the same direction. Results from the patient´s 3 months evolution post- transplantation will be presented.

Poster Board Number: 2598

GENE THERAPY AS A NEW APPROACH TO MITIGATE CUTANEOUS RADIATION SYNDROME: HYPE OR REALITY

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Cutaneous radiation syndrome (CRS) caused by accidental or therapeutic irradiations is characterized by iterative inflammatory waves, incomplete wound healing and poor revascularization. Local injection of Bone marrow-Mesenchymal Stem Cells (MSCs) or Adipocyte derived Stem Cells (ASCs) has been recently demonstrated to favour wound healing in animal models of CRS and in few patients. Paracrine processes are thought to be crucial in this context. Thus grafting modified MSCs/ASCs in order to transiently produce and secrete ad hoc factors may represent a pertinent strategy. Based on the literature, we chose to evaluate Sonic Hedgehog (Shh) as a first candidate. Shh is a morphogen involved in embryonic development. It codes for a protein which can induce proliferation of a variety of cell types including keratinocytes, neuronal precursor stem cells, hematopoïetic stem cells and also involved in angiogenesis. In a first step we set up an *in vitro* study model to evaluate the response of irradiated fibroblasts to conditioned media (CM) from ASCs secreting or not Shh. ASCs were electroporated (Amaxa®) with a plasmid coding for Shh and then were cultured in minimal essential medium with 10% fetal calf serum and fibroblast growth factor (2ng/mL) at 37°C in air with 5% CO2 for 7 days. At the end of the culture the different culture media were harvested. Minipig fibroblasts were *in vitro* irradiated at the dose of 25Gy gamma (1.5 Gy/min) and incubated within 10 min in the different CM. Fibroblasts were harvested at different times (30min, 6h, 24h and 48h post irradiation) and analyzed for apoptosis (Annexin V staining n=9 for each condition) and gene expression using RT-qPCR (n=3 for each gene). Globally, Shh significantly improve fibroblasts survival : apoptosis rate decreased in presence of Shh-ASC CM from 6 hours post irradiation to 24h ($p<0.05$). In addition we observed a sustained production of proangiogenic factors such as VEGF and ANGPT1 (at 24h post irradiation). Secondly we evaluated the biologic activity of Shh-ASCs in a NOD/SCID mice model. Shh-ASCs and ASCs were injected in BD Matrigel® "growth factor reduced". Then BD Matrigel® loaded or not with cells were subcutaneously injected in NOD/SCID mice (n=10 for each group). One week post implantation, mice were euthanized and BD Matrigel® were dissolved in 1ml of Drabkin lysis buffer. After homogenization, haemoglobin was quantified by spectrophotometry at 540nm. Interestingly the amount of haemoglobin was twice higher in Shh-ASCs-BD Matrigel® than in ASCs only BD Matrigel® ($p<0.05$). Work is going on to characterize the Shh-ASCs activity upon keratinocytes and determine in minipig model whether Shh transient gene therapy may represent a useful strategy to mitigate CRS (Exp. Hematol. 2010;38:945-956).

Poster Withdrawn

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Poster Board Number: 3002

HUMAN STEM CELLS FROM EARLY FETAL BLOOD, CHORION AND AMNIOTIC FLUID DIFFERENTIATE INTO PODOCYTES AND IMPROVE RENAL GLOMERULOPATHY IN ALPORT MICE.

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Alport Syndrome (AS) is a hereditary glomerulopathy, in which a mutation in COLIVA3, A4 or A5 chain genes impairs glomerular podocyte function, leading to compromised filtration and progressive glomerulosclerosis. AS patients experience hematuria, proteinuria and progress to end stage renal failure by the age of 20 to 40. Currently there is no cure, only the empiric treatments of renal dialysis or transplantation. To ameliorate AS, stem cells have been proposed as vehicles for gene or protein delivery because of their ability to expand, differentiate and repopulate a host *in vivo*. We investigated the potential of human fetal stem cells found in 1st trimester fetal blood and chorionic villi samples (CVS) and 2nd trimester amniotic fluid (AF), to differentiate into podocytes and improve renal glomerulopathy in a mouse model of AS. We first investigated the podocyte differentiation potential of the cells *in vitro*, either cultured on ColIV or co-cultured with wild type mouse glomeruli for 2 weeks. In both protocols, we found a significant up-regulation of the podocyte genes CR1, CD2AP, SYNAPTOPODIN, PODOCIN, NEPHRIN and VEGF along with a down-regulation of the progenitor genes CD24, CD133 and BMI-1. We next analysed the migration capacity of the cells towards Alport and wild type glomeruli using *in vitro* chemotaxis assays; cells from all sources migrated significantly more towards the Alport than control glomeruli *in vitro*. Finally, cells were transplanted into 7 week-old Alport or wild type mice and their engraftment and therapeutic effect assessed 2 weeks later. We observed that cells from all sources engrafted significantly more in Alport glomeruli, consistent with cell homing being enhanced in the presence of tissue injury. We also observed that transplanted Alport mice (n=10) maintained their weight for two weeks while 63% of non-transplanted control animals (n=10) dropped their weight below a 20% endpoint. In addition, transplanted Alport mice showed significantly lower blood urea levels compared to untreated animals (20.4±3.3, 27.5±1.0 mmol/l respectively, $P<0.01$). We conclude that early human fetal stem cells from fetal blood, chorion and amniotic fluid differentiate into podocytes and improve renal glomerulopathy in Alport mice.

Poster Board Number: 3004

FUNCTIONAL INDUCED HUMAN TROPHOBLAST STEM LIKE CELLS DERIVED FROM FIBROBLAST

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Placenta trophoblast stem (TS) cells differentiate into the cytotrophoblast and syntiotrophoblast in terminal villi, or become extravillous trophoblasts which invade the maternal decidua and remodel maternal terminal arteries. Abnormal placental cell differentiation and function in early pregnancy have been implicated with the development of preeclampsia and intrauterine growth retardation later in pregnancy. Currently, the *in vitro* study of human first trimester placenta development is limited by availability of primary trophoblast cultures and fidelity of immortalized trophoblast and choriocarcinoma cell lines. To address this need, we established human induced trophoblast stem (iTS) like cells by reprogramming somatic cells with a pool of transcription factors. iTS like cells similar as mouse TS cells form flat isolated colonies after passaged more than 60 days. In the study we reported here, we have analyzed genome-wide gene expression, epigenetic signature, and invasion capacity of these iTS like cells. First, iTS like cell and its somatic donor cell trancriptome was determined using Agilent human DNA array. 3953 genes are significantly up-regulated (more than 2 fold) in iTS like cells vs. donor fibroblast cells. Among these transcipts the stem cell renewal genes: TERT, Sall4, Klf5 etc; and TS related genes, such as Tead4 and Cdx2. The up-regulated genes suggested that the cell identity transformed from somatic cell into self-renewal trophoblast lineage. Secondly, we determined epigenetic signature of TS gene, ELF5, in iTS like cells by detecting DNA methylation status and mRNA. We found that in iTS like cells, Elf5 was DNA hypermethylated, whereas Elf5 mRNA was still expressed. These results suggest that Elf5 gene regulation by other mechanisms than DNA methylation. Thirdly, we have further examined differentiation ability of iTS like cells by studying their invasive capabilities (the character of EVT) and formation of multi nucleated cells (the character of ST). After 7 days differentiation, HLA-G positive cells and multinucleated cells sporadically stained (5 to 10%). The invasion capability of iTS like cells was determined by culturing cells on matrigel-coated transfer chambers and after 20~22hrs, the invasion percentage of iTS like cells vs. fibroblast was 44% vs. 26%. In conclusion, these results provide evidences that human iTS like cells harbor similar TS self-renewal and lineage specific transcripts. Further, iTS like cells have the capability to differentiate into invasive trophoblast like cells and form multinucleated giant cells.

Poster Board Number: 3006

REPLICATIVE SENESCENCE OF HUMAN PLACENTA-DERIVED MULTIPOTENT CELLS IS MEDIATED THROUGH PKC REGULATION

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Placenta-derived multipotent cells (PDMCs) are multipotent mesenchymal stromal cells (MMSC) isolated from human term placenta. These cells express bone marrow MMSC markers, are able to differentiate into cell types of all three germ layers *in vitro*, and are highly immunomodulatory. Moreover, being isolated from the placenta, PDMCs provide an easily accessible source of multipotent cells without ethical problems. However, similar to many adult stem cells (ASCs), PDMCs will senesce after a period of *in vitro* culture, which limits prevalent use of these versatile cells. We therefore focus on studying the mechanisms of PDMC senescence. Proliferation was attenuated after approximately 25 population doublings (PDs), and staining for β-galactosidase, a marker of senescence, was positive in late-passage PDMCs. Reactive oxygen species (ROS) detection revealed that the level of H2O2 was increased in late-passage PDMCs, presumably caused by up-regulation of the manganese superoxide dismutase (MnSOD). The cyclin dependent kinase (CDK) inhibitor p21 was found to be elevated in late-passage PDMCs accompanied with cell cycle arrest at G0/G1 phase. However, inhibition of H2O2 had no effect on p21 expression or cell cycle arrest. The tumor suppressor p53 is known to regulate the expression of p21, but p53 expression is decreased in late-passage PDMCs, and inhibition of p53 by the p53 inhibitor PFT-α did not alter the accumulation of p21. These findings suggest that p53 is not involved in the senescence of PDMCs. It has been reported that MAPK and protein kinase C (PKC) activation play important roles in p53-independent cell senescence mechanisms. We found that in late-passage PDMCs, phosphorylation of ERK was elevated.

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Inhibition of ERK attenuated the H2O2 level in late-passage PDMCs, however, the p21 level was not reduced by ERK inhibition. Expression of the PKC family member PKC-α was found to be elevated in late-passage PDMCs, and inhibition of PKC-α attenuated the accumulation of p21 in late-passage PDMCs. Furthermore, cell cycle arrest was also reversed by PKC-α inhibition. Our data shows that PDMC senescence involves the up-regulation of p21 and leads to cell cycle arrest. These events also involve PKC-α regulation in a p53- and ERK-independent pathway. The role of ERK activation and H2O2 accumulation in late-passage PDMCs is still unclear and consulting further investigation

Poster Board Number: 3008

HUMAN MESENCHYMAL STEM CELLS ISOLATED FROM UMBILICAL CORD AND PLACENTA EXERT IMMUNOSUPPRESSIVE ACTIVITIES THROUGH AN IDO-DEPENDENT MECHANISM.

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Mesenchymal stem cells derived from umbilical cord (UC-MSCs) and placenta (PL-MSCs) are considered to be adult stem cells that can be easily obtained in large quantities by a less invasive method in comparison to bone marrow-derived MSCs (BM-MSCs). However, the biological and immunoregulatory properties of UC-MSCs and PL-MSCs are still poorly characterized. Particularly; the underlying mechanisms of this immunomodulation remain undefined. Recent research demonstrated that BM-MSCs express the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), known to suppress T-cell responses. This study was designed to address whether IDO contributes to the immunoregulatory functions of UC-MSCs and PL-MSCs *in vitro*. To isolate UC-MSCs and PL-MSCs, umbilical cord and placenta were digested with collagenase and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. The expression profiles of several MSC markers were examined by flow cytometry. UC-MSCs and PL-MSCs from passage 3-5 were used for *in vitro* adipogenic and osteogenic differentiation assays by culturing in appropriate induction media. The results demonstrated that UC-MSCs and PL-MSCs were easily expanded to 18-20 passages while maintaining the undifferentiated state and exhibiting MSC markers CD73, CD90, and CD105 but do not express the hematopoietic markers CD34 and CD45. Similar to BM-MSCs, UC-MSCs and PL-MSCs were able to differentiate to several mesodermal-lineages including adipocytes and osteoblasts. Fascinatingly, immunomodulating properties of UC-MSCs and PL-MSCs were not difference from BM MSCs. In addidition, IDO secreted by UC-MSCs and PL-MSCs was responsible, at least in part, for induction of immunosuppressive activities in the same manner as BM-MSCs. This study supports the clinical application of UC-MSCs and PL-MSCs as therapeutic tool not only for regenerative medicine, but also for the treatment of GvHD after bone marrow transplantation.

Poster Board Number: 3010

INVESTIGATING THE ROLE OF EXTRINSIC AND INTRINSIC SIGNALS REGULATING HUMAN PLURIPOTENT STEM CELL SURVIVAL

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Human pluripotent stem cells (hPSCs), due to their capacity to differentiate into any cell type found in the body, have enormous potential for cell-based regenerative therapies. However, the realization of this potential remains hampered by many unanswered questions regarding the pathways that regulate self-renewal, survival, and differentiation of these cells. hPSCs, including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), exhibit poor survival upon dissociation, hindering the ability to generate scalable cultures and perform genetic manipulation on these cells. In a previous high-content screen for small molecules the modulate hESC survival we identified an inhibitor of Rho kinase (ROCK) that significantly enhances the survival of dissociated hPSCs. Recent literature has demonstrated that upon dissociation, hPSCs experience a ROCK-mediated terminal overactivation of the actin-myosin cytoskeleton, leading to a loss of cell integrity and apoptosis. While the cellular phenotypes associated with this apoptosis have been described, it is still unclear what signals are altered in hPSCs during dissociation that lead to actin-myosin hyperactivation. To examine this we have taken two approaches: evaluation of the extrinsic and intrinsic signals that are modified by dissociation, and a high content screening approach to identify novel small molecules capable of regulating hPSC survival. In the first approach, we have found that cell-cell signaling via E-cadherin is important for the survival of dissociated hPSCs as loss of E-Cadherin signals results in decreased survival upon dissociation. One key event after hPSC dissociation is relocation of E-cadherin away from the surface leading to loss of cell-cell junctions and subsequent cell blebbing and apoptosis. We hypothesize that dissociation of hPSCs leads to greater apoptosis due to internalization of and loss of signals from cell-cell junction proteins. One means to improve hPSC survival would then be via regulating endocytosis in order to control the cellular localization of cell-cell junction proteins including E-cadherin and integrins. This could reduce ROCK activation as a result of dissociation and subsequent cell death. In our second approach, we are continuing to screen libraries of known and novel small molecules to find additional regulators of hPSC survival. Novel molecules will be used to better characterize how hPSC survival can be regulated in response to dissociation. Improving our understanding of signals regulating hPSC survival will improve our ability to stably culture hPSCs and improve our ability to manipulate them for potential therapeutic use.

Poster Board Number: 3012

EXPOSURE OF FUNGAL VOLATILE ORGANIC COMPOUNDS CAUSES CYTOTOXICITY IN HUMAN EMBRYONIC STEM CELLS

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Microbial growth in damp indoor environment has been correlated with health risk. Although genotoxicity and cytotoxicity of some microbial volatile organic compounds (VOCs) including 1-octen-3-ol on lung epithelial cell line A549 has been reported, however; the toxicity of fungal VOCs on human embryonic stem cell lines has not been explored. This study was aimed to determine the cytotoxicity of 1-octen-3-ol, a major fungal volatile organic

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compound associated with mushroom and mold odors, on human embryonic stem cells (hESCs) in an attempt to determine the developmental toxicity of 1-octen-3-ol on growing embryo. 1-octen-3-ol exists as enantiomers, (R)-(-)-1-octen-3-ol and (S)-(+)-1-octen-3-ol, which exhibit identical physical and chemical properties but different three-dimensional orientation. We performed cytotoxic assays by exposing hESCS to racemic 1-octen-3-ol and its enantiomers, (R)-(-)-1-octen-3-ol and (S)-(+)-1-octen-3-ol using an airborne exposure technique assuming inhalation as the prime mode of delivery of 1-octen-3-ol in people residing in moldy buildings. Racemic and (S)-(+)-1-octen-3-ol exhibited greater cytotoxicity to hESCs than (R)-(-)-1-octen-3-ol. The Inhibition concentration 50 (IC50) values for these fungal VOCs were 40 to 80-fold lower than that of vapor phase toluene, an industrial chemical used as a positive control in this study. Our report pioneers modeling of human embryonic stem cells as an *in vitro* approach to screen the developmental toxicity of fungal VOCs associated with building-related illness and indicates that in the vapor phase, the fungal natural product 1-octen-3-ol and its enantiomers are more toxic than toluene to hESCs.

Poster Board Number: 3014

INDUCTION AND MONITORING OF INTERMEDIATE MESODERM FROM HUMAN INDUCED PLURIPOTENT STEM CELLS AND EMBRYONIC STEM CELLS

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The differentiation method from pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), into kidney lineage remains to be developed. Kidney is derived from one of early embryonic germ layers, intermediate mesoderm (IM), and directing pluripotent stem cells into IM lineage is a crucial step for kidney regeneration. Here we have efficiently generated human iPSC lines that contain an allele of OSR1 gene, a marker for IM, into which a green fluorescence protein (GFP) gene was knocked-in by homologous recombination. We have also established an induction protocol using combinational treatment of growth factors, in which differentiation of human iPSCs produced cultures consisting of more than 40 % OSR1+ cells. Furthermore, the cells expressed other IM marker genes, and could differentiate into multiple cell types included in IM derivative organs, such as kidney, gonad and adrenal cortex *in vitro*. These results suggest that our differentiation protocol can induce human pluripotent stem cells into IM cells with similar developmental potential to that in embryos, supplying systems for understanding the developmental mechanisms of IM lineage and cell sources for the regeneration of mature IM derivatives.

Poster Board Number: 3016

EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY LIGANDS DIFFERENTIALLY MODULATE SINGLE-CELL MIGRATION IN SUBPOPULATIONS OF PRIMARY HUMAN MULTIPOTENT STROMAL CELLS

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Factors that drive migration and trafficking of bone marrow-derived multipotent stromal cells (MSCs) *in vivo* are still poorly understood, yet it is desirable to control these behaviors for therapeutic applications. We and others have reported that ligands of the EGFR family can control the migration, proliferation, and trafficking of bone marrow-derived multipotent stromal cells (MSCs), processes important for therapeutic applications. Human MSCs express three members of the epidermal growth factor receptor (EGFR) family -- EGFR/HER1, HER2, and HER3 - that are implicated in bone development, homeostasis, and healing *in vivo*, and that regulate cell migration and motility in several cell types. In particular, Neuregulin (NRG) has been shown to have a protective effect against hypoxia conditions in mouse derived MSCs, but to our knowledge there are no studies that seek to understand the role of NRG/HER-3 signaling axis in human MSC migration. Therefore, in this study we investigate whether motility of human MSCs is regulated by EGF or Neuregulin (NRG) activation of EGFR family members, and whether subpopulations within the MSC culture differentially respond to these ligands. We analyzed behavior of primary human MSCs migrating on a defined fibronectin-coated glass in the basal and EGF or NRG stimulated states, using time-lapse video microscopy of individual cells. The 12-hr migratory tracks of individual cells were generated using a Brownian motion tracking algorithm. Means and associated standard errors were calculated from individual cells' speed, total migratory path length, and net displacement for a given experimental condition. Cell morphological parameters were determined and correlated with a cell's migration statistics to derive population distributions. Basal cell motility and sensitivity to growth factor stimulation were correlated with cell projected area, and evidence of EGFR family autocrine stimulation in the motility response was observed.

Poster Board Number: 3018

CHARACTERISATION OF HUMAN SALIVARY GLAND STEM CELLS TO RESCUE RADIATION-INDUCED SALIVARY GLAND DAMAGE

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Each year, 600,000 patients are diagnosed worldwide with head and neck cancers, and will be treated with a combination of (chemo) radiation therapy. A large proportion of these patients will develop severe xerostomia ('dry mouth syndrome'), as a consequence of salivary gland ablation. In mice, we showed previously that transplantation of c-Kit+ stem/progenitor cells (SSCs) isolated from cultured salivary gland samples, rescued irradiated mice from xerostomia(1). We currently aim to characterise putative stem cells residing within the human gland, as a possible therapeutic avenue for the treatment of radiation-induced xerostomia in humans. We initially show by immunohistochemistry that cells expressing c-kit can be localized to the larger ducts within the intact biopsy-derived human salivary gland. Salispheres cultured from human salivary gland tissue also express c-kit and cytokeratin-5, proposed salivary gland stem cell markers. When dispersed and

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analysed by flow cytometry, 0.10 % (± 0.08 % S.D.) of salisphere-derived cells were c-kit+. Salisphere-derived cells also express the established adult stem cell marker proteins CD24, CD49f and CD133 at differing frequencies (CD24: 19.65 % ± 21.70 S.D.; CD49f: 16.92 % ± 20.22 S.D.; CD133 17.98 % ± 18.28 % S.D.), suggesting that the cultures potentially contain stem cell populations, and may be therapeutically useful. In order to become useful in the clinical environment, putative salivary gland stem cells must demonstrate an ability to differentiate into functional cells of the salivary gland. During *in vitro* culture, salispheres originally negative for differentiation markers gradually obtained expression of the salivary-gland associated proteins α-amylase, aquaporin-5 and mucin-5, as shown by immunohistochemistry and PCR. The ability of the cells to proliferate and differentiate in an *in vivo* setting will also be determined. After a period of eight weeks, kidney capsules of immune-compromised mice engrafted with whole spheres, dispersed cells and c-kit+ cells showed morphological resemblance to human salivary gland tissue. In addition, amylase and Muc-5 positivity was observed by immunohistochemistry, suggesting that salivary stem cells (SSC) have an innate potential for differentiation into saliva-producing cells. Finally, we correlated our *in vitro* tissue culture findings with donor age, and found that donor tissue from patients older than 50 yielded significantly less salispheres in culture. Interestingly, earlier data of ours indicated that greater patient age at time of radiotherapy is positively correlated with the likelihood of xerostomia development. Considered together, the present clinical and *in vitro* data suggest that a causal relationship exists between the quality or quantity of SSCs and presentation in the clinic of xerostomia. The data also imply that these SSCs, if manipulated correctly *in vitro*, could provide a future strategy for the largely unmet clinical need for xerostomia therapy.

Poster Board Number: 3022

CHARACTERISATION OF RODENT SALIVARY GLAND STEM CELLS

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Salivary glands are often exposed to radiation, during radiotherapy for head and neck cancers. This may result in life-long salivary gland impairment, severely reducing the quality of post-treatment life of patients. Stem cell therapy may be an option to prevent radiation-hyposalivation. In mice, we recently showed that transplantation of salisphere-derived c-Kit+ stem/progenitor cells (SSCs) rescued the salivary glands from radiation damage. The aim of current study is to characterise the exact stem cells in the salivary glands of rodents, which can regenerate the radiation damaged salivary gland. To obtain putative stem cells dissociated salivary glands of mice and rats were cultured to form three-dimensional aggregates of cells termed salispheres. To investigate the ability of these rat and mouse spheres to differentiate into cells/structures of their respective salivary gland cells *in vitro*, we plated single spheres in collagen matrigel matrix. When supplemented with different growth factors, such as R-spondin with insulin, keratinocyte growth factor (KGF) and the rock inhibitor, the spheres developed into duct like structures, which stained immunohistochemically positive for duct cell markers, such as CK14, and CK18. Furthermore, the matrix stained positive for Periodic Acid Schiff (PAS) indicating secretion of acinar cell derived salivary gland mucins. In matrigel without supplements, salisphere derived single cells were able to regrow spheres for at least 10 passages. These results provide evidence that the salispheres may contain stem cells, with self-renewal potential and able to form the structures of the salivary gland. To characterize the exact stem cells in mice salispheres derived from submandibular tissue cells, single cells from the trypsinised salispheres were stained with flourochrome labelled antibodies specific for certain known stem cell markers. Flow cytometrical analysis showed that these salispheres contain, 0.65% ±0.93 c-Kit+, 56.12%±9.40 CD24+, 80.04% CD29+, 55.2%±15.8 CD49f+, and 5.42%±3.62 CD133+ cells. Moreover, 0.02%±0.02 of all cells are double positive for CD24/c-kit, 32.71%±12 for CD24/CD49f, 55.46±11.45 for CD24+/CD29+, but surprisingly no overlap was found between CD133 and c-Kit-positive cells. To investigate the regenerative potential of the cells, we transplanted salisphere derived, CD133+ or CD24+/CD29+ or CD24+/c-kit+ cells, intraglandularly into the locally irradiated (15 Gy) salivary gland of mice. To investigate potential salivary gland specific genes, microarray studies on 4d salispheres were performed, revealed upregulation of, MyoD, BMP7, Cdh1, Cdh13, Notch1 and CD44 in comparison with mouse salivary glands and fibroblats. Confirmation of their upregulation and importance in salisphere stem cells is in progress. Conclusion - Salispheres do contain cells with stem cell activity, as determined by prolonged *in vitro* growth, *in vitro* differentiation and *in vivo* regeneration. Further investigation of subpopulations of double and triple stem cell marker positive cells may reveal the true identity of the salivary gland stem cell in rodents.

Poster Board Number: 3024

RENOPROTECTIVE ASSESSMENT OF USSC TRANSPLANTATION IN NUDE MICE WITH ACUTE KIDNEY FAILURE

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Background: Stem cells have been vastly investigated for treatment of kidney failure during the recent years and promising results have been obtained with different sources such as bone marrow derived cells, embryonic cells, and tissue specific stem cells. However, in spite of protective effects on other disease models, the renoprotective potential of human umbilical cord unrestricted somatic stem cells (USSC), have not been examined so far. Methods: In the present study, acute kidney failure was induced in female nude mice by subcutaneous injection of 14 mg/Kg cisplatin (day 0). After 24 hours, the mice received either 0.5 ml phosphate buffered solution (PBS) containing 105 USSC or 0.5 ml PBS alone. The mice were sacrificed in day 4 and serum and kidney samples were obtained for assessment of urea, creatinine, and histopathologic state. Results: The serum urea level of USSC group was higher than the control group (268.8±59.1 and 99.3±13.6, respectively; p-value<0.05) and histopathologic examinations revealed that the severity of tissue damage was higher in USSC-receiving mice in comparison to control mice (138.9±8.7 and 112.6±7.0 respectively; p-value<0.05) In addition, the number of hyaline casts per high power field was more in USSC group (16.5±8.5 and 8.8±1.2 respectively; p-value<0.05). Conclusion: Unexpectedly, our results indicate that, transplantation of USSC following cisplatin induced nephropathy, has a negative effect on kidney function and structure and probably USSC is not a suitable source for cell therapy of acute kidney injury.

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FRESHLY AND CULTURED AMNIOTIC FLUID STEM CELLS RESTORE THE SATELLITE CELL NICHE IN A MOUSE MODEL OF SPINAL MUSCULAR ATROPHY

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Background: Mutations in the survival of motor neuron gene (SMN1) are responsible for spinal muscular atrophy (SMA), a fatal neuromuscular disorder. Using a Cre-LoxP system, mice carrying a homozygous deletion of Smn exon 7 directed to skeletal muscle (HSA-Cre, SmnF7/F7 mice) present the main clinical features of human progressive muscular dystrophies. Cell–mediated therapies are still limited by the inefficiency of progenitors suitable to functional muscle regeneration. We have investigated whether amniotic fluid stem (AFS) cells are able to functionally restore muscle tissue. Methods: Freshly isolated and expanded mouse AFS cells GFP+ were injected via tail vein in 3 months old HSA-Cre, SmnF7/F7 mice. Long term (15 months) morphological analyses and secondary transplantation were performed to demonstrate the restoration of satellite cell niche. Results: Muscle tissue of surviving AFS cells treated animals was analyzed 15 months after cell injection. Morphological analyses demonstrated that treated muscle tissue was compact, with few central nucleated fibres (<1%), comparable to WT mice. Moreover, 58% of myofibres were GFP+ in AFS cells treated mice. PCR analyses of GFP expression performed 15 months after transplantation on three different muscles (gastrocnemius, tibialis anterior and diaphragm) confirmed that the AFS cells had spread throughout the body engrafting in various skeletal muscle tissues. Finally, freshly isolated satellite cells from muscles of AFS cells transplanted primary hosts were injected into muscles of HSA-Cre, SmnF7/F7 secondary hosts. One month after secondary transplantation, all the mice in the AFS cell group had GFP+ fibres (31%). Conclusions: This is the first study demonstrating the functional integration of non-muscle and non-embryonic stem-derived cells into the satellite cell niche. The AFS cells' ability to replenish the skeletal muscle stem cell niche could make them a promising treatment option for muscular dystrophy.

Poster Board Number: 3028

LINEAGE SPECIFIC DISTRIBUTION OF HIGH LEVELS OF GENOMIC 5-HYDROXYMETHYLCYTOSINE IN MAMMALIAN DEVELOPMENT.

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Methylation of cytosine is a DNA modification associated with gene repression. Recently, a novel cytosine modification, 5-hydroxymethylcytosine (5-hmC) has been discovered. Here we examine 5-hmC distribution during mammalian development, adult tissues and derivative cellular systems commencing with lineage specification at the blastocyst stage. We show that the developmental dynamics of 5-hmC are different from those of 5-mC; in particular 5-hmC is enriched in embryonic contexts compared to adult tissues. Levels of 5-hydroxymethylcytosine are high in cells of the inner cell mass (ICM) in blastocysts, and co-localise with nestin-expressing cell populations in mouse post- implantation embryos. Compared to other adult mammalian organs, 5-hmC is strongly enriched in bone marrow and brain, wherein a high 5-hmC content is a feature of both neuronal progenitors and in contrast with other contexts, postmitotic neurons. We show that high levels of 5-hmC are not only present in mouse and human embryonic stem cells (ESCs) and lost during differentiation, as it has been reported previously but also reappear during the generation of induced pluripotent stem cells (iPSC); thus 5-hmC enrichment correlates with a pluripotent cell state. Our findings suggest that apart from the cells of neuronal lineages, high levels of genomic 5-hmC are an epigenetic feature of embryonic cell populations and cellular pluri- and multi-lineage potency. To our knowledge, 5-hmC represents the first epigenetic modification of DNA discovered whose enrichment is so cell-type specific.

Poster Board Number: 3030

A ROLE FOR GATA3 IN CYCLING HEMATOPOIETIC STEM CELLS

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Gata3 is a zinc finger transcription factor known to play multiple roles in development and in the specification of various adult tissues including skin, hair follicle and mammary gland. Although Gata3 transcript expression has been described in highly purified fractions of hematopoietic stem cells (HSC), neither protein-level expression nor evidence for any functional role have been reported. We analysed Gata3 protein expression in highly purified populations of Long-Term (LT)-HSC by immunofluorescence microscopy. Gata3 protein was detected in 30% of RholoKit+Sca+Lin-CD49blo (RKSLCD49blo) and 50% of Kit+Sca+Lin-CD150+CD48b- (KSLCD150+CD48-) cells, but not in the newly described Intermediate-Term-HSC fraction (RholoKit+Sca+Lin-CD49bhi). Interestingly, RKSLCD49blo and KSLCD150+CD48- fractions contain 30% and 50% of biologically active LT-HSC respectively. Confocal microscopy localized Gata3 in the cytoplasm of quiescent LT-HSC. However, after culture with cytokines, Gata3 had relocated entirely to the nucleus. This finding predicted that a functional role for Gata3 might be demonstrable only in cycling HSC. In order to show conclusively that Gata3 expression was restricted to LT-HSC rather than other contaminating cell types present in the RKSLCD49blo and KSLCD150+CD48- fractions, we made use of a Gata3 mutant mouse in which GFP is expressed under control of the endogenous Gata3 promoter. When marrow from these mice was fractionated according to GFP fluorescence and tested in recipient mice, long term engraftment was obtained only from the GFP+ fraction, while all transient engraftment activity remained in the GFP- fraction. To analyse Gata3 function we used a conditional Gata3 deletant mouse strain crossed to Mx1-Cre deleter mice. Gata3 excision had no effect on steady-state hematopoiesis and did not affect the frequency of phenotypic (RKSLCD49blo) HSC. However, when Gata3-deleted cells were transplanted into irradiated hosts where they would be induced to cycle, their reconstituting capacity was dramatically enhanced relative to wild-type competitors. When cultured with cytokines, Gata3-/- LT-HSC entered active cell cycle with expected kinetics but thereafter cycled more slowly than Gata3+/+ cells. After 7 - 14 days in culture, the long-term competitive *in vivo* repopulating activity of Gata3+/+ cells was lost as expected. In striking contrast, the reconstituting activity of cultured Gata3-/- cells increased beyond the activity initially placed into culture. These results are the first to implicate Gata3 in regulating key HSC properties, demonstrating a suppressive role in self-renewal of dividing HSC, presumably in favour of differentiation. Endogenous Gata3 is thus identified as a significant modulator of the outcome of cell divisions uniquely in the HSC subset capable of long-term maintenance of the self-renewal function.

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Poster Board Number: 3032

CONJUNCTIVAL POLYPLOID CELLS AND DONOR-DERIVED MYOFIBROBLASTS IN OCULAR GRAFT-VERSUS-HOST DISEASE

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Purpose: After allogeneic hematopoietic stem cell transplantation (allo-SCT), ocular graft-versus-host disease (GvHD) is a common complication, typically symptoms being dry eye syndrome with features of fibrosis. In this study, we have identified and quantified two cell types - myofibroblasts (MFB) and polyploid cells - in the conjunctival surface of allo-SCT patients (pts), and have explored their kinetics and association with local and systemic GvHD. Methods: Imprint cytologies were obtained by pressing the conjunctival surface with a sterile, non-abrasive cellulose acetate filter (Millipore). After retraction, typically a monolayer of the outermost cells of the epithelium were retrieved. MFB were identified by immunofluorescent (IF) staining for alpha-smooth muscle protein (αSMA). Polyploid (PP) cells were detected by aberrant chromosome content analyzed via X/Y-FISH. In female pts with a male donor (M→F group), donor genotype were be identified by sex chromosome detection using FISH methodology. IF and FISH methods were applied *in situ* on the same filter, and amounts of MFB and PP cells are expressed as percent of all cells on the filter. In all, seventy samples from 46 pts were obtained 1-122 months after allo-SCT. Results: The total myofibroblast density (MFBTOT) was higher in allo-SCT pts compared to healthy controls, and increased by time elapsed since transplantation ($p<0.001$). In M→F recipients this increase proved to due to a significant ($p<0.001$) gradual elevation of donor-derived MFB (MFBXY), whereas recipient-derived MFB (MFBXX) did not vary over time. Clinical ocular GvHD correlated with MFBXY/MFBTOT ratio ($p=0.034$). No association between MFBTOT or MFBXY and systemic or ocular GvHD was observed. In the MtF group (n=25), 28 of 37 imprints (76%) both MFBXY and MFBXX were detected. In pts >36 months post-transplant, 11/12 imprints, a median of 9.4% (1.4-39) MFBXY and 3.6% (0-11) MFBXX was found. In one patient, 1.6% MFBXY were detected at three weeks post-transplant. Polyploid cells (6-24n), exclusively of recipient origin, were found to a median of 0.6 (0-37)%. The PP cell density differed significantly ($p<0,001$) between time intervals with a maximum, 8.9 (0-35)% of all cells, at 3-12 months. No correlation between polyploid cells and GvHD (ocular or systemic) was observed. ConclusionS: The MFB has been indicated as a culprit in chronic inflammation and fibrosis. The observations that (i) MFBTOT and time after transplantation; and (ii) MFBXY/MFBTOT ratio, correlated with ocular GvHD, suggest a role of MFB in GvHD pathogenesis. The constant finding of recipient-derived MFBXX cells many years after transplant in pts with 100% donor hematopoiesis, indicates that there is a non-hematopoietic differentiation route to MFB. The origin and role of polyploid cells after allo-SCT remains obscure.

Poster Board Number: 3034

WDHD1 MODULATES THE POST-TRANSCRIPTIONAL STEP OF THE CENTROMERIC SILENCING PATHWAY

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The centromere is a highly specialized chromosomal element that is essential for chromosome segregation during mitosis. Centromere integrity must therefore be properly preserved and is strictly dependent upon the establishment and maintenance of surrounding chromatin structure. Here we identify WDHD1, a WD40-domain and HMG-domain containing protein, as a key regulator of centromere function. We show that WDHD1 associates with centromeres in a cell cycle-dependent manner, coinciding with mid-to-late S phase. WDHD1 downregulation compromises HP1α localization to pericentric heterochromatin and leads to altered expression of epigenetic markers associated with this chromatin region. As a consequence, such reduced epigenetic silencing is manifested in disrupted heterochromatic state of the centromere and a defective mitosis. Moreover, we demonstrate that a possible underlying mechanism of WDHD1's involvement lies in the proper generation of the small non-coding RNAs encoded by the centromeric satellite repeats. This role is mediated at the post-transcriptional level and likely through stabilizing Dicer association with centromeric RNA. Collectively, these findings suggest that WDHD1 may be a critical component of the RNA-dependent epigenetic control mechanism that sustains centromere integrity and genomic stability.

Poster Board Number: 3036

DETECTION AND QUANTIFICATION OF MRNA TRANSCRIPTS IN SINGLE EMBRYONIC STEM CELLS BY OPENARRAY® AND DIGITAL PCR

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There is increasing evidence that pluripotent embryonic stem cell cultures (ESCs), once thought to be a uniform cell type, are in fact highly heterogeneous, especially in terms of RNA expression. Until recently, understanding the dynamics of both miRNA and mRNA expression within a cell population has been severely limited by the lack of an efficient and effective means to assay gene expression within individual cells. Here we demonstrate our success using the Taqman™ chemistry on Open Array platform (Life Technologies, Inc) to easily and robustly analyze miRNA and mRNA expression in single embryonic stem cells. Open Array results are consistent with our established single cell analysis platform, confirming the robust reproducibility of the Open Array system, and establishing the technology required for high throughput single cell profiling experiments. In our most recent studies we confirm highly heterogeneous expression of key developmental control genes in ESC and demonstrate heterogeneous expression of several miRNAs, adding a new dimension to our understanding of cell type complexity in ESC cultures. In addition, we utilized cutting-edge digital PCR (dPCR) technology to quantify the number of transcripts present in each cell, providing an unprecedented level of resolution to single cell transcript analysis. Single mouse embryonic stem cells (mESCs) were collected via FACS sorting into 96 well plates containing Single Cell-to-Ct® Lysis Buffer with subsequent DNA treatment, RT and preamplification performed using either gene-specific or miRNA Megaplex® primers and samples were sent to Life Technologies for analysis. In total we analyzed the expression of 64 mRNA transcripts in 48 single cells and 61 miRNAs in 27 cells using the Open Array® platform. For

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dPCR, cells were lysed following FACS and sent to Life Technologies where RNA was reversed transcribed and the entire RT reaction was applied to the Open Array®. dPCR was used to quantify Nanog and Gapdh transcripts for pools containing between 1-20 cells and per-cell transcript levels were consistent across all samples. Our experience with the Open Array® platform and its dPCR capability has dramatically increased our throughput, providing a flexible and cost-effective means of analyzing gene expression in single cells without compromising the reliability and sensitivity of our assay.

Poster Board Number: 3038

ENHANCED STEMNESS DUE TO EMBRYONIC MICRORNA

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Stem cells hold promise as an unlimited source for various clinical and biotechnological applications. Speculating the regulatory mechanism in Stem cells will greatly enhance the understanding of stem cells as well as their applications. Recent advances in small RNA research have implicated microRNAs (miRNAs) as important regulators of development and differentiation. miRNAs constitute a large family of non-coding small RNAs of approximately 22 nucleotides (nt) in length which interfere in gene expression either by inhibiting translation or RISC mediated mRNA degradation. The expressions of miRNAs are often regulated in tissue specific and developmental stage manners. Studies have shown that a group of microRNAs are expressed only in hES cells including mir371 cluster. The mir-372 microRNA (miRNA) is expressed specifically in human embryonic stem (ES) cells, and quickly decreases after cell differentiation and proliferation. Therefore, mir-372 was investigated as one of the key factors essential for maintenance of ES cell renewal and pluripotency in this study. The lenti-viral based miRNA expression system was used to transgenically transduce the mir-372 into human mesenchymal stem cells (MSCs). The mir-372 - transducted cells expressed exceptional key ES cell markers, Nanog and Rex1. Cell proliferation assay and other findings will need to be further explored and will be announced later. At this stage we can conclude that mir-372 not only functions to maintain the pluripotent state of human embryonic stem cells but also function to manage the same in Mesenchymal stem cells (MSCs) which may offer a great chance for therapeutic intervention.

Poster Board Number: 3040

WNT β-CATENIN SIGNALING INDUCES AGING OF BONE MARROW MESENCHYMAL STEM CELLS THROUGH DNA DAMAGE AND P53 P21 PATHWAY

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Recent studies have demonstrated importance of cellular extrinsic factors in aging of adult stem cells. This study examined effects of the aged serum on aging of bone marrow mesenchymal stem cells (MSCs) and explored mechanisms of aging of MSCs mediated by Wnt/β-catenin signaling. 10% serum from young (3 ~ 4 weeks) or old (64 ~ 72 weeks) SD rats was added into the culture medium for MSCs. Senescence-associated changes of the cells were examined with SA-β-galactosidase staining and ROS staining. The proliferating ability was detected in MTT assay. The survived and apoptotic cells were determined by AO/EB staining and Hochest 33342 staining. Old rat serum (ORS) promoted MSC senescence and reduced proliferation and survival of the cells. The expression of β-catenin and GSK-3β were detected by immunofluorescence and western blotting. The results showed that the Wnt/β-catenin signaling of the cells was activated after ORS treatment. In order to evaluate roles of Wnt/β-catenin signaling on MSC aging, we examined aging, proliferation and survival of MSCs after modulating Wnt/β-catenin signaling. The results indicated that continuous exposure to 100ng/ml Wnt 3a induce senescence and dysfunction of MSCs incubated with young rat serum (YRS). Senescence and dysfunction of MSCs in the medium contained ORS could be reversed by Wnt/β-catenin signaling inhibitor DKK1 or β-catenin siRNA. For further exploring mechanisms of Wnt/β-catenin signaling on the MSC aging, we detected expression of γ-H2A.X, a molecular marker of DNA damage response, p16INK4a, p53 and p21 by reverse transcription-PCR, immunofluorescence and western blotting. The results showed the expression of γ-H2A.X, p16INK4a, p53 and p21 increased in senescent MSCs induced with ORS or following a Wnt 3a exposure in YRS. This study indicates that Wnt/β-catenin signaling pathway may play a critical role in MSC aging induced by serum of the aged animals and suggests that the DNA damage response and p53/p21 pathway may be main mediators of MSC aging induced by excessive activation of Wnt/β-catenin signaling.

Poster Board Number: 3042

A GENOME-WIDE PROFILING OF SILENCED INSERTS TARGETED BY PIGGYBAC, TOL2, AND SLEEPING BEAUTY TRANSPOSONS IN HEK293

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Most of the advanced gene trap systems currently available are retrovirus-based gene trap vectors that favor inserting into active genes. Consequently, the inactive chromosomal regions are either hard or unable to be targeted by these vectors, leading to the targeting profile of retrovirus-based gene trap vectors biases toward the active gene category in undifferentiated ES cells. To overcome the aforementioned obstacles encountered by current gene trap vector systems, a novel gene trap system, enabling to target both active and silenced regions in mammalian genome in an effective and random manner is urgently needed. Unlike retrovirus-based vectors, preferably targeting to the active gene regions, DNA transposons, such as piggyBac, Tol2, and Sleeping Beauty display a relatively random targeting profile. In this study, we aim to explore the potential of developing a DNA transposon-based gene trap system enabling to target both active and epigenetically silenced genes in the mammalian genome. To this end, here we conducted a side-by-side genome-wide target profiling of piggyBac, Tol2, and Sleeping Beauty with focuses on the targets located in silenced regions of the HEK293 genome. We observed that the chromosomal integration rate of piggyBac and Tol2 are 94% and 95%, respectively, once they are delivered into cells. And the transgene in 19% of piggyBac and 24% of Tol2 targeted HEK 293 clones are silenced. Furthermore, except for the 19% silenced clones, most of the piggyBac targeting clones expressed transgene (hygromycin resistant gene) at a moderate to high level. On the contrary, only about one half of Tol2 targeted clones expressed sufficient hygromycin resistant gene product for drug selection. Plasmid rescue experiments are now undergoing to retrieve the chromosomal sequences flanking the piggyBac and Tol2 silenced targets. The similar evaluation on Sleeping Beauty is also undergoing.

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Poster Board Number: 3044

IDENTIFICATION OF TISSUE SPECIFIC STEM / PROGENITOR CELLS IN AUDITORY PATHWAY

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Objects: Recently, in the region of regenerative medicine, it focused on the tissue specific stem/progenitor cells such as neural stem cells in adult brain that has multipotency and identified these cells in various organs. The treatment for the deaf patients that have the auditory damages has been tried, however it was difficult to heal completely. In this research, in order to find out the keys of treatments for these patients by using regenerative medicine, we tried to isolate stem cells from auditory pathway and identify the cell characterization and gene expressions. Methods and Results: 3 days age of mice were injected BrdU to the hypodermic at 2 times/day for 3 days continuously, and sacrificed after 16 weeks to identify the slow-cycling cells suggesting the possibility of stem cells. The results of immunohistochemistry showed that a few cells were identified as BrdU+ and ABCG2+ double positive cells in the section of cochlear nuclei in auditory pathway. Furthermore, we assumed that it was possible to purify stem cells as side population (SP) cells. Cochlear nuclei cells were isolated from 6 weeks age of mice and stained with Hoechst 33342. After staining, SP cells were sorted as a negative fraction. The population of SP cells was about 4% of total cells of cochlear nuclei. Furthermore, we analyzed the gene expression of SP cells by RT-PCR. The results showed that some specific markers of stem/progenitor cells, such as Oct-3/4, Sox2 and ABCG2, were over-expressed in SP cells compared with main population (MP) cells. Cell suspension from CN were cultured in the conditioned medium, sphere formation was found. Now we are going to study whether these cells have the multipotency as stem/progenitor cells, and analyze the specific gene function that identified by microarray analysis.

Poster Board Number: 3046

DEVELOPMENT OF POTENTIAL BIOMARKERS FOR TUMORIGENESIS IN EMBRYONIC STEM CELL BASED THERAPY

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Embryonic stem cells (ESCs) give rise to the possibility that stem cell therapy. However, before cell replacement therapies can be used, the problems with the tumorigenicity of ESCs must be solved. In the present study, we attempt to find biomarkers related with tumor formation of ESCs. We induced neuronal differentiation from H9, a hESCs, using a five-stage method and performed characterization of hESCs in each developing stages. We decided stage 3 as the step of cell sorting and performed separating cells on SSEA3 and CD133 using the MACS. In the results of *in vivo* assay with each sorted cells, CD133 positive and negative cells were not related with tumorigenesis. But groups injected the SSEA3 positive cells showed high levels of tumor forming rate as 100%, otherwise the group injected SSEA3 negative cells not detected tumorigenesis during periods of study. In these results, we recognized that SSEA3 related with undifferentiated status has important role in reduction of tumor formation on hESCs tumorigenesis, suggesting that these appears to be a useful biomarker for the assessment of the safety of stem cell-based therapies using hESCs.

Poster Board Number: 3048

INVESTIGATING THE EFFECT OF HIGH GLUCOSE ON BONE MARROW-DERIVED MESENCHYMAL STEM CELLS TOXICITY: ROLE OF APOPTOSIS

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Hyperglycemia which occurs under the diabetic condition induces a number of serious complications such as cell death in various types of cells. Mesenchymal stem cells (MSCs) are population of cells with ability to differentiate into various cell types. Despite of intensive investigation on effect of high glucose poisoning on many cell types, there is no report on high glucose-induced toxic effect on MSCs. The goal of this study was to investigate the cytotoxic effect of high glucose on rat bone marrow derived MSCs toxicity and also to examine the involvement of Bax, pro-apoptotic, Bcl-2, anti-apoptotic, and caspase-3, the executioner of apoptosis, protein expression, during high glucose-induced apoptosis in MSCs. The cell viability was measured by MTT assy. Exposure to high glucose significantly decreased the level of cell proliferation in a dose-dependent manner. The expression of pro-apoptotic Bax and anti-apoptotic Bcl2 and caspase-3 protein expressions were measured by western blotting analysis. High glucose at 60 mg/ml enhanced over expression of pro-apoptotic Bax, as well as caspase 3-protein compared to the controls, while the expression of Bcl-2 protein did not change significantly. The Bax/Bcl2 ratio significantly increased in high glucose-treated cells. Finally, it may be concluded that high glucose could cause toxicity of MSCs, in which apoptosis by mitochondrial pathway plays an important role.

CHROMATIN IN STEM CELLS

Poster Board Number: 3050

TEMPORAL CHANGES OF HISTONE MODIFICATIONS DURING DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

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Embryonic Stem (ES) Cell differentiation is accompanied by overall changes in chromatin accessibility regulated in epigenomic layers. To follow this dynamic process, we differentiated mouse embryonic stem (mES) cells into mesendoderm cells (precursors of endoderm and mesoderm) using a serum free medium with Activin A. Analysis of the expression of 10 lineage-specific marker genes showed strong enrichment of mesendoderm population after 6 days of differentiation. Changes of epigenomic states were assayed with chromatin immunoprecipitation followed by DNA sequencing (ChIP-Seq) in three time points (day 0, 4 and 6). We found the distribution of the histone modification H3K27me3 was temporally altered upon differentiation. In mES cells, H3K27me3 was highly enriched in the genomic neighborhood of lineage-specific developmental regulatory genes and depleted near pluripotency regulatory genes. During differentiation, the enrichment near some lineage markers was gradually lost, and it was enhanced near pluripotency regulatory genes.

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Poster Board Number: 3052

CELL FATE SPECIFICATION OF CARDIAC PROGENITOR CELLS AND STEM CELLS BY DEFINED FACTORS IN MICE

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Congenital heart defects (CHDs) are the most common birth defects in human, occurring in approximately 1% of all live births, and heart disease remains a leading cause of death globally. For patients with extensive damage to heart muscle, treatment options are very limited and there is no therapy available to reverse the damage once it has occurred. Recently, therapies based on cardiac progenitor/stem cells have emerged as promising potential cardiac therapeutics. This offers a new approach to treat patients with heart disease, including the potential to differentiate into new heart muscle and thus reverse dysfunction. However, to take advantage of regenerative therapy, we need to develop efficient and reliable ways to induce and differentiate cardiac projenitor/stem cells. Our strategy is to directly induce/differentiate Isl1+/Wt1+ embryonic cardiac progenitors and Sca1+/c-Kit+ adult stem cells by identifying and manipulating their master regulators. Recently, I discovered key epigenetic factors that act as master regulators for cardiomyocyte induction in a mammalian system (Takeuchi & Bruneau 2009). Interestingly, additional epigenetic genes belonged to the family of the factors had a specific function in heart cell specification. Based on these findings, we profiled epigenetic genes expressed in cardiac progenitor/stem cells. We identified 15 genes that are highly enriched in these cells and investigated their function in mice. Strikingly, a gene deletion caused loss of outflow tract formation as in Islet1-deleted mouse embryos. Flow cytomety revealed that cells positive for the deleted genes were also positive for Isl1 (50%), cKit (40%) and Sca1 (10%). This suggests that these genes may potentiate induction/differentiation of cardiac progenitor/stem cells. These candidate genes were co-expressed with cKit/Sca1 in the adult heart. Importantly, their overexpression resulted in ectopic expression of cardiac progenitor genes *in vivo*. Our study will elucidate the fundamental mechanisms by which cardiac progenitor/stem cells are induced and differentiated into specific lineages, which will be applicable for future cell-mediated heart therapeutics.

Poster Board Number: 3054

EED-DEPENDENT GLOBAL AND LOCAL CHROMATIN STATES IN PLURIPOTENT AND DIFFERENTIATING MOUSE EMBRYONIC STEM CELLS

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The molecular programs determining pluripotency and differentiation of embryonic stem (ES) cells underlie epigenetic regulation. The chromatin of ES cells is characterized by a hyperdynamic state, open conformation, overall elevated gene expression levels and bivalent domains, which are defined by the concomitant presence of activating histone H3K4 tri-methylation (me3) and repressive histone H3K27me3 marks. Bivalently modified chromatin is thought to keep genes in a transcriptionally poised state. While Trithorax group proteins are involved in H3K4 methylation, the methylation of H3K27 is catalyzed by the Polycomb repressive complex 2 (PRC2), consisting of the core subunits EZH2, SUZ12 and EED. The deletion of EED leads to the loss of EZH2 and SUZ12 protein expression and thus results in a global loss of H3K27 methylation. EED knockout (KO) ES cells are reported to express bivalently regulated genes at higher levels than wildtype (wt) ES cells while the non-bivalently marked pluripotency-associated genes are expressed at levels comparable to wt ES cells. We aim to understand the role of EED in the organization of global nuclear chromatin architecture. Further we study the role of PRC2 during *in vitro* ES cell differentiation. By micrococcal nuclease digestion we observed similar chromatin accessibility in wt and EED KO ES cells, indicating that the absence of the H3K27 methylation does not result in a global opening of ES cell chromatin structure. In agreement with this, FRAP analyses revealed similar linker histone dynamics in wt and EED KO ES cell nuclei. By fluorescence microscopy we could, however, show that DAPI-/H3K9me3-positive heterochromatic foci were more defined, larger in size and less frequent in EED KO ES cells as compared to wt ES cells. Further, global H3K9me3 levels were slightly decreased, pointing towards a PRC2-mediated crosstalk between H3K9 and H3K27 methylation, and the enzymes that set or read these marks. Currently performed genome-wide H3K9me3 ChIP-seq analyses using wt and EED KO ES cell chromatin preparations will provide further information on the genomic regions affected by PRC2-dependent H3K9 methylation. To investigate the role of PRC2 during ES cell differentiation, we induced cardiomyocytic differentiation in wt and EED KO ES cells. The data revealed severe defects in EED KO ES cell differentiation as they failed to form contracting cardiomyocytes and retained more cells with colony formation potential after an extendend period of time under differentiation culture conditions. In comparison to wt cells the expression of pluripotency-associated genes remained high in EED KO cells during this period. Together, we report that the nuclear H3K9me3-enriched heterochromatin is reorganized in EED KO ES cells and that pluripotency-associated genes require PRC2-mediated silencing upon *in vitro* differentiation.

Poster Board Number: 3056

THE ROLE OF CHROMATIN MODIFYING ENZYMES IN CHROMATIN RE-ORGANIZATION DURING MOUSE EMBRYONIC STEM CELL DIFFERENTIATION

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In Embryonic Stem cells (ES) derived from the E3.5 embryo, the chromatin is uniformly and widely dispersed whereas in Epiblast Stem Cells (EpiSCs) derived from the E5.5 embryo, compact chromatin domains are observed. ES and EpiSCs are both pluripotent stem cells but unlike ES cells, EpiSCs are primed-for-differentiation and unable to produce a chimera mouse. ES and EpiSCs are reversibly convertible *in vitro*. Following differentiation of ES cells, the global chromatin architecture is extensively re-organized including the formation of large compact chromatin domains. These observations led to our hypothesis that an open chromatin structure is required for stem cell pluripotency and that chromatin re-organization is critical in the early differentiation program. The question we wish to address is what is the mechanism responsible for global chromatin changes observed upon the reversible conversion between ES and EpiSCs and in ES cell differentiation? In other words, what change in chromatin organization is inherent in lineage commitment? For this purpose, we have obtained a number of ES cell lines that are null mutants including a component of the nucleosome remodelling and histone deacetylation complex NuRD, the DNA methyltransferases 3a/b and a histone methyltransferase. We convert these mutant ES cells to EpiSCs and take advantage of a transmission electron microscopy technique, called electron spectroscopic imaging, which allows us to observe a change in the global chromatin configuration with high resolution and contrast. The absence of epigenetic modifiers crucial in establishing heterochromatin domains in early development leads to defects in chromatin reconfiguration in ES/EpiSC conversion.

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Poster Board Number: 3058

CULTURE-INDUCED DNA DEMETHYLATION OF THE EPIGENOME OF HUMAN EMBRYONIC STEM CELLS

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The genetic and epigenetic integrity of human embryonic stem cells (hESCs) is critical to their future application in research and medicine. hESCs cultured in serum free media can accumulate point mutations, aneuploidy, and progressive epigenetic changes over prolonged culture *in vitro*. For the first time, we previously identified ascorbate as the only molecule in the very widely used Knock-out serum replacement medium that is sufficient to induce widespread and remarkably specific and consistent DNA demethylation of 1,847 genes in both HES2 and HES3 cells, including CD30, a member of the tumour necrosis factor receptor superfamily and a biomarker for embryonal carcinoma cells. The underlying biochemistry and the upstream molecular mechanism of ascorbate-induced DNA demethylation remain to be determined. We speculate that ascrobate directly changes DNA topology by affecting the activity of histone demethylases and thus modifying the accessibility of DNA methylases and/or demethylases. Our preliminary data show that ascorbate leads to increased global histone acetylation, decreased H3K9-methylation as well as increased expression of Jumonji histone demethylase JMJD2 in hECSs. Notably, JMJD2 (2A, 2B, 2C, 2D) demethylate H3K9 me3/me2, which is required for the binding of the repressive protein HP1 and is associated with heterochromatin formation and transcriptional repression in a variety of species. We propose that addition of ascorbate in culture media triggers JMJD2-mediated H3K9 me3/me2 demethylation, subsequently leading to genome wide DNA demethylation in hESC epigenome. Using CD30 as a marker, we are currently assessing the effect of enforced overexpression of JMJD2B and JMJD2C on CD30 expression in hESCs.

Poster Board Number: 3060

NON-HISTONE CHROMATIN PROTEINS AND PROTOONCOGENES HMGA1 AND HMGA2 ARE GUARDIANS OF HUMAN EMBRYONIC STEM AND TUMOR CELL GENOMES

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Background: Besides critical roles for high mobility group AT-hook (HMGA) proteins in early mouse and human development, clinicians have known for some time that high HMGA levels are associated with most malignant human neoplasias such as breast cancer, sarcomas, pancreas and lung cancer. In fact, HMGA genes are the only known proto-oncogenes coding for DNA architectural chromosomal proteins consistently over-expressed in nearly all types of naturally occurring cancers. Their expression level correlates directly with the degree of malignancy and metastatic potential. In line with these observations, recent microarray analyses showed that both HMGA genes belong to a small class of genes whose expression is critical to the cancer phenotype in cells that carry two key oncogenic mutations in p53 and Ras. Intriguingly, HMGA2 is also found in rare tumor-inducing cells (cancer stem cells) which are resistant to chemotherapy. Findings and Conclusions: We recently demonstrated that HMGA1 and HMGA2 possess intrinsic abasic (AP) and dRP site DNA cleavage activities. By employing a variety of cell-based assays, we provided evidence that HMGA2 promotes cellular resistance against DNA damage that is targeted by base excision repair pathways, and that this protection directly correlates with the level of HMGA2 expression. We demonstrate here that HMGA2 has an additional DNA-stabilizing role at stalled replication forks in human ES and cancer cells, thus substantially increasing genome stability after spontaneous or induced DNA damage. These observations reveal another unexpected and crucial function for HMGA2 in genome stability in human ES and cancer (stem) cells. Funded, in part, by BMRC Singapore.

Poster Board Number: 3062

CHROMATIN DYNAMICS DURING CARDIOVASCULAR DIFFERENTIATION FROM HUMAN EMBRYONIC STEM CELLS

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Directed differentiation of human embryonic stem cells (hESCs) into definitive cardiovascular cells provides a model system for studying the molecular mechanisms of human cardiovascular development. In this study, we analyzed cells undergoing cardiovascular directed differentiation at five key developmental stages: pluripotency, mesoderm, tripotential cardiovascular progenitor (KDR+/PDGFRα+), cardiac committed and definitive cardiovascular cells. We performed whole-transcriptome analysis using traditional microarray to monitor changes in gene expression over the course of differentiation. To gain insight into the mechanisms of gene regulation during cardiovascular differentiation, we sought to examine chromatin dynamics over time. We performed chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) for histone H3 lysine 4 trimethylation (H3K4me3), H3K27me3 and H3K36me3, an activating modification, a repressive modification and a surrogate for RNA polymerase, respectively. In addition, we utilized DNase hypersensitivity analysis to identify cis-regulatory elements genome-wide. Many known cardiac transcription factors, including NKX2.5 and TBX5, had high levels of H3K27me3 early in differentiation that decreased over time, concurrent with increasingly levels of H3K4me3, H3K36me3 and RNA expression. This highlights the importance of repressing expression of transcription factors until the precise time when their activity is developmentally appropriate. In contrast, structural proteins involved in cardiac muscle contraction, such as alpha myosin heavy chain (MYH6) and cardiac troponin T (TNNT2), were not marked with H3K27me3 at any stage of development, but showed the same increase in H3K4me3, H3K36me3 and RNA expression as cardiac transcription factors. Of note, several cardiac transcription factors showed the highest levels of H3K4me3, H3K36me3 and RNA expression at the tripotential cardiovascular progenitor stage of differentiation. These include the closely-related forkhead transcription factors FOXC1 and FOXC2, which have been implicated in cardiovascular defects in both mice and humans, and are thought to be involved in second heart field development. Thus, our data can be used to identify the specific stage(s) of differentiation regulated by known cardiac transcription factors. In addition, we have identified several transcription factors with a less well-defined role in cardiogenesis that have similar chromatin patterns to transcription factors known to be critical to cardiac development. These include zinc finger proteins, homeobox-containing transcription factors and nuclear receptors. Thus, our results show that genome-wide chromatin state profiles facilitate the discovery of novel regulators of key developmental processes. Our ongoing work is focused on knocking down these genes during cardiovascular directed differentiation from hESCs and zebrafish embryogenesis to characterize their role in heart development.

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Poster Board Number: 3064

CHROMATIN PROTEOMICS IN EMBRYONIC STEM CELLS REVEALS A ROLE FOR CHROMATIN REMODELERS IN STEM CELL IDENTITY

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Chromatin in ES cells has an open structure that is permissive to the transcriptional machinery. Upon differentiation, chromatin is reorganized into a more closed, restrictive structure. In order to identify the proteins that regulate chromatin biology in ES cells, we developed an assay (DCAP: Differential Chromatin Associated Proteins), which enables us to reveal ES cell-specific chromatin associated proteins by Multidimensional Protein Identification Technology (MudPIT). We identified several chromatin remodeling proteins which are exclusively associated with chromatin in undifferentiated ES cells, including Smarcd1. Reduced levels of Smarcd1 in ES cells lead to elevated levels of acetylated histone H3 (H3ac) and reduced levels of heterochromatin protein 1 alpha (HP1α), implying a transition to a hyperactive chromatin state. Smarcd1 Knock-Down (KD) derived embryoid bodies (EBs) display elevated levels of Oct4, suggesting delayed differentiation. The formation of neuronal progenitor cells (NPCs) from Smarcd1-KD cells is perturbed, indicating the importance of Smarcd1 for neuronal differentiation. Taken together, these results show that Smarcd1 acts to restrict chromatin in differentiating ES cells, reducing pluripotency during early differentiation.

Poster Board Number: 3066

LOSS OF HISTONE DEMETHYLASE LSD1 NON-AUTONOMOUSLY CAUSES GERMLINE STEM CELL TUMORS IN THE *DROSOPHILA* OVARY

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Specialized microenvironments called niches keep stem cells in an undifferentiated and self-renewing state. Dedicated stromal cells form niches by producing a variety of factors that act directly upon stem cells. The size and signaling output of niches must be finely tuned to ensure proper tissue homeostasis. While advances have been made in identifying factors that promote niche cell fate, the mechanisms that restrict niche cell formation during development and limit niche signaling output in adults remain poorly understood. Here we show that the histone demethylase Lsd1 regulates the size of the germline stem cell (GSC) niche in *Drosophila* ovaries. GSC maintenance depends on BMP signals produced by a small cluster of cap cells located at the anterior tip of the germarium. Lsd1 null mutant ovaries carry small germline tumors, containing an expanded number of GSC-like cells that display ectopic BMP signal responsiveness away from the normal niche. Clonal analysis and cell-type specific rescue experiments demonstrate that Lsd1 functions within the escort cells (ECs) that reside immediately adjacent to cap cells and prevents them from ectopically producing niche specific signals. Temporally restricted gene knock-down experiments suggest that Lsd1 functions both during development, to specify EC fate, and in adulthood, to prevent ECs from forming ectopic niches independent of changes in cell fate. Further analysis shows that Lsd1 functions to repress dpp expression in adult germaria. The role of Lsd1 in regulating niche specific signals may have important implications for understanding how disruption of its mammalian homolog contributes to cancer and metastasis.

Poster Board Number: 3068

THE ROLE OF KAP1 IN NEURAL STEM CELLS

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Epigenetics is becoming an important puzzle piece to gain a better understanding for several diseases. It is characterized by the interplay of genetic background, environmental influences and aging. Epigenetics enables the ability of adjusting to the environment and being flexible without intervening DNA. Kap1 (also nown as TIF1β or TRIM28) is a KRAB-associated protein and shows the ability of being a universal co-repressor of gene-transcription. By using a NestinCre - loxP approach we have generated mice with a CNS-specific Kap1 deletion. We have found that Kap1-NestinCre knockout mice die before or around birth. To clarify this finding we have generated neurospheres derived from KAP1-deficent embryos and found that the lack of Kap1 has no influence on neural stem cell proliferation and self-renewal. Furthermore the Kap1 knockout neurospheres showed the ability to differentiate into neurons and astrocytes. The result of histochemical staining showed that the brain morphology of Kap1 knockout embryos at E13.5 does not differ compared to wild type controls. In summary we have found that although Kap1 knockout embryos do not show any anomalies in development until E 13.5, Kap1-NestinCre knockout mice either die before or after birth. We are currently further investigating the mechanisms behind this lethality.

Poster Board Number: 3070

THE D4Z4 INSULATOR PROTECTS RETROVIRUS TRANSGENES FROM SILENCING IN PLURIPOTENT STEM CELLS

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A challenge for genetic manipulation of pluripotent stem cells is to maintain long-term expression of transgenes. Indeed, many transgene vectors, retroviral vectors included, are often silenced in transgenic animals, stem cells and also in induced pluripotent stem cells (iPS). It has been shown that silencing of the reprogramming factors delivered by retroviral vectors is required during reprogramming, and is one of the critical criteria to distinguish fully and partially reprogrammed clones. In addition, transcriptional silencing of retrovirus vectors in cells is a major obstacle to current gene therapy approaches. Insulator elements can overcome this problem by protecting transgenes from position effects. The best studied insulator, the chicken ß-globin locus (HS4) insulator, has to date had limited effectiveness in viral vectors. A novel insulator has been discovered within the D4Z4 repeat sequence that is involved in human Facio-scapulo-humeral dystrophy (FSHD). Protection from silencing is dependent on CTCF and A-type lamin binding at the 5' end of D4Z4. We investigate here whether D4Z4 is able to protect retrovirus vector transgenes from silencing in mouse embryonic stem cells and induced pluripotent stem cells. We analyze the expression from self-inactivating retroviral vectors (HSC1) bearing an EF1α-EGFP-ires-puro reporter over 5 months of culture. We observed that the unprotected vector is silenced over time (30% of cells are GFP positive without selection pressure versus 99% under puromycin selection). We were able to remove this expression extinction by inhibiting G9a activity to decrease di-methylated H3K9 levels on the proviral DNA. The HS4 insulator partially protects the HSC1 retrovirus (70% versus 30% eGFP positive cells with or without HS4 respectively), but silencing still occurs. We compared these vector versions (unprotected vector or insulated by cHS4 insulator in the LTRs) to retroviral vectors with D4Z4 subfragments

cloned between gag and the EF1α promoter. We observed that the 3′ D4Z4 subfragment protects provirus from silencing in all cells, while the 5′ D4Z4 subfragment does not although it decreases the variability of fluorescence intensity in those cells that do express. Only by combining the 3′ D4Z4 element with HS4, is silencing prevented and consistent levels of expression maintained. Bisulfite sequencing 150 days after infection shows proviral DNA is hypomethylated when it contains D4Z4, which correlates with a higher frequency of expression. Our results demonstrate that D4Z4 is the first insulator capable of maintaining long-term, stable expression of retrovirus vectors in pluripotent stem cells. We propose that D4Z4 may induce an active chromatin structure on the provirus independent of the integration site. We are comparing provirus expression at identical integration sites in the absence and presence of D4Z4. We are also evaluating the ability of D4Z4 retroviral vectors to maintain expression of transgenes designed for cell marking and suicide vector delivery in iPS cells.

Poster Board Number: 3072

MECHANISMS REGULATING CHROMATIN PROTEIN DYNAMICS IN EMBRYONIC STEM CELLS

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Embryonic stem cells (ESCs) are characterized by unique epigenetic features including decondensed chromatin, hyperdynamic association of proteins with chromatin and a permissive transcriptional program. Here we investigate the mechanisms that regulate chromatin plasticity in ESCs. Using epigenetic drugs and mutant ESCs lacking various chromatin binding proteins, we find that histone acetylation enhances chromatin dynamics specifically in euchromatin, while histone H3 lysine 9 (H3K9) methylation and lamin A expression restrict chromatin dynamics exclusively in heterochromatin. In contrast, we find that DNA methylation and nucleosome repeat length have little or no effect on the dynamics of chromatin-binding proteins in ESCs. Altered chromatin dynamics was associated with perturbed ESC differentiation. Together, these data delineate the mechanisms responsible for chromatin plasticity in ESCs, and indicate that the epigenetic state of the genome modulates chromatin plasticity and the differentiation potential of ESCs.

iPS CELLS

Poster Board Number: 3074

COMPARISON OF THE PHENOTYPES OF DIFFERENTIATION AND MOLECULAR SIGNATURES IN HUMAN EMBRYONIC STEM AND INDUCED PLURIPOTENT STEM CELLS

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Human embryonic stem (ES) cells show some variation in differentiation propensity, and induced pluripotent stem (iPS) cells may also. To apply ES/iPS cells for any purposes such as drug discovery, studies on disease mechanism and cell therapy, we need to select the clones which can differentiate into target cells completely with no remaining of undifferentiated cells as early as possible. To characterize the clones, we established iPS clones from fibroblasts derived from people with various age and sex and performed colony formation assay and neural differentiation by modified serum-free floating culture of embryoid body-like aggregates (SFEBq) methods. To explore the factors which are related to the differentiation propensity, we compared gene/miRNA expression and methylation status between these clones. These data indicate that molecular signatures of ES cells and iPS cells are very similar and to predict the differentiation propensity by molecular signatures is difficult.

Poster Board Number: 3076

HUMAN IPS CELLS PRODUCED USING GMP-COMPLIANT METHODS PRODUCE FUNCTIONAL NEURONS

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Induced pluripotent stem cells (iPSCs) have an enormous potential for the treatment of a wide range of diseases and injuries. But there is an exceptional regulatory challenge for stem cell derived products as they represent complex biological materials. One of the challenges is the derivation and differentiation of human iPSCs without the use of xenogeneic materials (xeno-free) under current good manufacturing practice (cGMP) protocols. Undefined components in the media formulation lead to difficulty in quality control and may cause lot-to-lot variability. We have established a cGMP compliant laboratory and established protocols and cGMP compliant standard operating procedures (SOPs) for the derivation of xeno-free somatic cells from human tissues and have used them to reprogram the somatic cells into iPS cells under xeno-free and feeder-free conditions. The iPS cells derived were karyotyped, characterized using standard immunocytochemistry markers, and studied for their tri-lineage potential. We found that they are with normal karyotype; express cell surface markers Nanog, Tra 1-81 and SSEA-1 and are capable of multi-lineage differentiation *in vivo* as teratomas. Furthermore, we differentiated the GMP iPSCs into neuronal precursor cells and neurons under xeno-free conditions. To our knowledge, this is the first description of the generation of xeno-free terminally differentiated neurons proceeding from patient biopsies through pluripotent intermediates. These protocols and SOPs will serve as a platform for further advancement of the iPS technology for clinical translation and may aid to establish norms for cellular therapy using iPS cells.

Poster Board Number: 3078

EPIGENETIC DIFFERENCES BETWEEN HUMAN PLURIPOTENT STEM CELL LINES

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Pluripotent stem cells (PSCs) have unique transcriptional regulatory networks and epigenetic state to maintain their pluripotency. This study was carried out to test epigenetic variations between human PSC lines such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). For that, gene expression and histone modifications were examined between two hESC lines and four hiPSC lines by real-time PCR and chromatin immunoprecipitation (ChIP) analysis, respectively. Transcriptional levels of pluri-

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potency marker genes (OCT4, SOX2, NANOG, and REX1), iPSC-inducible factor genes (cMYC and KLF4), and developmental genes (SOX17, T, and NESTIN) varied between respective hESC and hiPSC lines. Regardless of the expression level of those genes, their promoter regions represented different patterns of permissive (H3K4me3 and H3K9ac) and repressive histone modifications (H3K27me3, H3K9me2, and H3K9me3) between human PSC lines. Bivalent histone modifications retained in promoters of developmental genes in both hESC and hiPSC lines. These results indicate that respective PSC lines may have a distinct epigenetic signature to regulate the transcriptional network for pluripotency.

Poster Board Number: 3080

PRE-EVALUATED SAFE HUMAN IPS CLONE DERIVED NEURAL STEM CELLS PROMOTED FUNCTIONAL RECOVERY AFTER SPINAL CORD INJURY WITHOUT TUMORIGENECITY IN ADULT COMMON MARMOSETS

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Recently, we have reported the effectiveness of transplantation of mouse as well as human iPS derived neural stem cells (NSCs) for spinal cord injury (SCI) in rodents. The purpose of this study is to determine the effectiveness and safeness of transplantation of human iPS derived NSCs (human iPS-NSCs) into the injured spinal cord in non-human primates, as a final stage of pre-clinical research. We used a pre-evaluated safe iPS clone, and induced their differentiation into NSCs using original protocol. A moderate contusive SCI was induced at the C5 level in adult common marmosets using a weight-drop device as described previously. 9 days after the injury, human iPS-NSCs at a density of 1000000 cells/5μl in medium without growth factors were transplanted into the injured spinal cord. Behavioral analyses were performed according to previous reports (original open field scoring scale (MIKY score), bar grip test, cage climbing test) until 12 weeks after SCI. Axial sections of spinal cords were subjected to histological analyses to determine the effects of transplantation. Grafted human iPS-NSCs that were survived and differentiated into NeuN positive neurons and GFAP positive astrocytes. It was noteworthy there was no tumor formation at least for 3 months after transplantation. LFB staining showed a significant decrease in demyelinated areas at the epicenter in the transplantation group compared to the vehicle-control group. Furthermore, quantitative analysis of the CaMK-IIα positive areas that there was a significant difference in CaMK-IIα positive areas at the epicenter between the transplantation and vehicle control groups. All of the examined behavioral tests also revealed that human iPS-NSCs promoted the functional recovery after SCI in adult common marmosets. Taken together, pre-evaluated safe human iPS clone derived NSCs could be a potential cell source for SCI in clinic.

Poster Board Number: 3082

DELAYED POST-STROKE RECOVERY BY EPIDURAL TRANSPLANTATION OF LATE-STAGE NEURAL PROGENITORS FROM HUMAN INDUCED PLURIPOTENT STEM CELLS VIA A UNIQUE SECRETOME

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Induced pluripotent stem cells (iPSCs) from over-expression of transcription factors in adult fibroblasts are promising source for tailor-made cell therapy in devastating stroke beyond therapeutic time window. However, risk of tumorigenesis is a major obstacle to iPSCs translational implantation. Here we show therapeutic potential of human iPSCs-derived late-stage neural progenitor/precursor cells (iPSC-NPCs) in murine stroke models through bystander effects after delayed extra-cerebral epidural engraftment. We first compared neuroprotective effects of different human stem cell types utilizing a trans-membranous co-culture system with rat cortical cells subjected to oxygen-glucose deprivation (OGD). In contrast to mesenchymal stem cells (MSCs) from bone marrow or umbilical cord Wharton's jelly, iPSC-NPCs and iPSCs preferentially enhanced neuronal survival and axonal growth as well as mitigated reactive astrocytosis. Notably, the gene and protein expression profiles of iPSC-NPCs and iPSCs showed a unique secretome from which we identified five enriched neuroprotective cytokines (BMP7, CXCL14, FGF8, FGF9, and IGFBP2) in the OGD model. Neutralization of the five cytokines significantly abolished neuroprotective effects in the iPSC-NPCs co-cultures. Moreover, we investigated one week-delayed iPSC-NPCs transplantation via epidural fibrin glue upon the infarct neocortex after permanent middle cerebral artery occlusion (MCAO) in adult rats. From 10 days post-transplantation onwards, the cell-transplanted rats showed significant sensorimotor improvement of the paretic forelimb as compared to the vehicle-transplanted rats. At four weeks after MCAO, the cell-transplanted rats had markedly reduced infarct size associated with ameliorated microglial infiltration and astrogliosis, while increased peri-infarct cortical angiogenesis and subcortical myelination preservation as compared to the controls. Very few iPSC-NPCs migrated across the dura and survived at the peri-infarct cortex while persistent functional recovery, suggesting beneficial paracrine effects from the grafted iPSC-NPCs. There was no tumor formation in any transplanted rat until four weeks post-MCAO. The results suggest that delayed epidural engraftment of iPSC-NPCs via topical fibrin glue can safely induce potent neuroprotection and functionally regenerative response in adult peri-infarct cortex through paracrine effects.

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Poster Board Number: 3084

TRANSPLANTATION OF HUMAN IPS CELL-DERIVED NEUROSPHERES FOR THE TREATMENT OF SPINAL CORD INJURY IN NOD-SCID MICE

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Induced pluripotent stem (iPS) cells have the potential to resolve the ethical issues and immunological rejection associated with embryonic stem (ES) cells. Recently, we have reported the effectiveness of transplantation of mouse iPS cell-derived neurospheres (iPS-NS) for spinal cord injury (SCI) in mice. In the present study, we performed the transplantation of human iPS-NS into injured spinal cord of NOD-scid mice to examine their therapeutic potential and safeness as a cell source of transplantation for SCI.
We established the directed neural differentiation of human iPS cells, using the cell line generated from adult human dermal fibroblast by retroviral transduction of four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) (Takahashi et al., 2007). Human iPS-NS were mainly differentiated into neurons *in vitro*. Adult female NOD-scid mice were used in this study. After laminectomy, contusive SCI was induced at Th10 level using IH impactor. Nine days after injury, the mice were to receive randomized human iPS-NS (n=28) or PBS (n=26). Motor functions in the hindlimbs had been assessed until 56 days after SCI by BMS, Rotarod test and Treadmill gait analysis using DigiGait system. Motor-evoked potential (MEP) was performed to evaluate the functional recovery in the mice after each intervention electrophysiologically. Grafted human iPS-NS survived and differentiated into neurons, astrocytes and oligodendrocytes in the injured spinal cord. We found that the most of human iPS cell-derived neurons were GABAergic neurons, and also found the synapse formation between human iPS cell-derived neurons and host neurons. We also observed human iPS-NS promoted axonal regrowth, which was demonstrated by the quantitative analyses of Neurofilament (NF-H), 5-hydrosytryptamine (5-HT) and growth-associated protein-43 (GAP43) positive fibers. Moreover, 5-HT positive fibers extended along with the human iPS cell-derived astrocytes. It is known that angiogenesis has the positive effect for tissue repair in the injured spinal cord, and we observed the transplantation of human iPS-NS promoted angiogenesis. We found that the human iPS cell-derived astrocytes secreted VEGF, and considered it was the reason for promotion of angiogenesis observed in the human iPS-NS group. As a result of these positive effects, the human iPS-NS group exhibited significantly better functional recovery than the control group. Consistently, MEP waves were detected in most of the iPS-NS group, whereas they were not detected in the control group. It is important to address safety issues about transplantation of human iPS-NS. To investigate the long-term safeness about the grafted iPS-NS, we extended follow-up period up to 112 days after SCI. Human iPS-NS grafted mice maintained their functional recovery and none of iPS-NS grafted mice showed teratoma or other tumor formation. The present study demonstrates the human iPS-NS could have potential benefits for SCI.

Poster Board Number: 3086

IMPACT OF VIRAL TRANSGENE EXPRESSIONS ON THE DIFFERENTIATION OF HUMAN IPS CELLS

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Induced pluripotent stem (iPS) cells can be generated from somatic cells by ectopic over-expression of four transcription factors, Oct4, Klf4, Sox2 and c-Myc. Recent findings suggest that factor-induced reprogramming primarily generates an undifferentiated intermediate cell state that is not completely pluripotent. These partially reprogrammed cells are characterized by the continued expression of viral transgenes, the incomplete expression of pluripotent genes and down-regulation of somatic cell marker genes. To quickly determine whether an iPS clone is a fully reprogrammed colony or a partially reprogrammed intermediate, a multiplex real-time RT-PCR assay was developed to detect the expressions of the viral Oct-4 transgene and the endogenous ES/iPS cell pluripotent gene, Nanog. Using this assay, the kinetics of viral transgene expression during cell differentiation was also monitored. Somatic cell reprogramming of human foreskin fibroblasts was accomplished using a polycistronic cre-excisable lentivirus expressing all four transcription factors. Because the four transgenes are transcribed as a single mRNA molecule, a single transgene analysis of viral Oct4 would represent the gene expression level of the whole cassette. Using this assay, we show that the viral transgenes were silenced upon the creation of human iPS cells, indicating full reprogramming. However, upon differentiation into either mesenchymal or neural stem cell progenitors, the viral transgenes were reactivated and viral expression levels remained high even after a two week differentiation protocol into specific cell lineages such as osteocytes. The efficiency of differentiation was significantly reduced in human iPS (hiPS) compared with human ES cells undergoing identical differentiation protocols, suggesting that the effects may primarily be due to conflicting pluripotency and differentiation signals rather than differences in genetic backgrounds and epigenetic stages. We are currently exploring ways to remove the transgenes using cre-excision and analyzing whether removal of the viral transgenes would restore the differentiation potential of post-excised hiPS cells to levels similar to those observed in human ES cells. The dynamic state of viral transgene expressions in hiPS and their differentiated progenies underscore the need for assays to quantitatively monitor gene expressions along with tools for the excision of the viral transgenes.

Poster Board Number: 3088

INDUCED PLURIPOTENT STEM CELLS A SLIPPERY SLOPE FOR NEURODEGENERATIVE DISEASE MODELING?

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The recent breakthrough in reprogramming somatic cells has invigorated the prospect that disease mechanisms that underpin various human diseases particularly the neurodegenerative disorders could be unraveled by using the disease-specific pluripotent stem cells. A number of studies have demonstrated that such disease-specific induced pluripotent stem cell (iPSC) could be generated relatively easy. Some recent studies have substantiated the utility of this technology in describing the initial characterization of neurodegenerative patient-derived iPSC as a proof of concept. However, as it is becoming evident now that the cell type of origin influences the molecular and functional properties of derived iPSC. The indications that reprogramming may erase the cell memory also raises the question if the disease phenotype may not be correctly represented or also erased in iPSC unless coaxed by further perturbation *in vitro* culture conditions. Other associated difficul-

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ties in iPSC research such as culture variability, selective adaptation of such cultures and the lack of robust protocols to generate homogeneous population of desired cell type may have compounding affects in the use of these cells in disease modelling. We have generated the human induced pluripotent stem cells (iPSCs) from a sporadic female patient with clear clinical symptoms of Alzheimer's disease (AD). Phenotypically, the fibroblasts and AD-iPSCs are indistinguishable from control fibroblasts, iPSCs and hESCs, however genome-wide transcriptomic analysis revealed a small number of genes that were significantly and differentially (Fold change > 1.5) expressed between control and disease populations of fibroblasts but the same were reset to ground state after reprogramming. Further analysis of differentially expressed genes in disease fibroblasts/Ad-iPSCs revealed biological functions that were related to neurogenesis, neuronal maturation, nervous system development etc. as determined by gene ontology (GO) enrichment. This may suggest that altered neuro-biological activities during their development into neurons. There seem no differences in AD-iPSCs and controls in neurosphere production and gene and protein expressions, further studies are in progress. While generation of AD-iPSCs, may serve as a potential *in vitro* model for the study of Alzheimer's disease, but issue of epigenetic modifications during reprogramming may remain an issue to be resolved. Unless this and other issues as above are addressed properly the prospects of iPSC in disease modelling may remain a slippery slope. Key Words: induced pluripotent cells. Neurodegenerative diseases. Disease modeling.

Poster Board Number: 3090

HUMAN INDUCED PLURIPOTENT STEM CELLS DERIVED FROM ALZHEIMER'S PATIENT SHOW ABERRATIONS OF LATE NEURAL DEVELOPMENT

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Alzheimer's disease (AD) manifests clinically as a loss of cognition including memory and other cognitive functions. The pathological characteristics of AD include neuronal and synaptic loss, activated microglia, gliosis and the pathognomonic entities - intraneuronal neurofibrillary tangles and the extracellular neuritic plaques. Currently, the study of AD is limited by a lack of model systems that can reproduce the precise sequence and timing of cellular and molecular events. Animal models offer reasonable phenocopies of AD, but suffer because comparative genomic and anatomical differences are prominent in relation to the human brain. Human induced pluripotent cells (hiPSCs) that now can be derived from somatic cells have the inherent ability to recapitulate human development *in vitro*. hiPSCs generated from individuals with AD arguably contain the genetic predisposition to AD that could provide valuable insights into the pathophysiology of that disease. We produced hiPSCs from a skin sample of a patient with early-onset AD and from a control subject, and also used hESC in this study. First, we demonstrated a stepwise differentiation process that mimicked the early stages of neural development in humans to promote the generation of neuroprogenitors. Both the hESCs and hiPSCs were initially cultured in an optimized feeder-free condition, which offered an efficient formation of cell aggregates. To initiate neuroectodermal specification, cell aggregates were differentiated in a defined neural induction medium to develop into neuroepithelia and neural rosettes-like structures that resembled the radial arrangements of columnar cells in the neural tube. It is of interest that there were no morphological differences observed between the AD-hiPSCs, hiPSCs-control and hESCs in the formation of neuroprogenitors. Quantitative real-time PCR analysis between these cells further indicated no differences in the expression levels of PAX6 and SOX1 genes, suggesting similar neural-differentiation efficiency. Subsequently, neural rosette structures were dispersed to form free-floating neurosphere-like structures, which can be propagated further or differentiated to mature neuronal subtypes. The adherent neurospheres on laminin-coated surface were direct differentiated to midbrain and forebrain lineage commitment using defined morphogens (FGF8, SHH, cAMP, ascorbic acid and IGF) and neurotrophins (BDNF and GDNF). Early differentiation demonstrated clear morphological differences between AD-hiPSCs and controls with former consistently producing poor neurons-like outgrowths in cultures. Further molecular and immuno staining studies are in progress to confirm whether AD-hiPSCs-derived neurospheres are capable of developing into mature functional neurons.

Poster Board Number: 3092

HUMAN INDUCED PLURIPOTENT STEM CELLS SHOW AN INTRINSIC PROPENSITY TO REVERT TO PLURIPOTENCY AFTER DIFFERENTIATION.

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Several studies have established that human induced pluripotent stem cells (iPSC) exhibit strong similarities with human embryonic stem cells (hESC), with regard to morphology, expression of pluripotency markers and functional properties such as embryoid body or teratoma formation. However, more detailed examinations of directed differentiation into specific lineages using several iPSC lines have shown that iPSCs responded to a lesser degree and with marked variability to chemical inducers of differentiation compared with hESC. Moreover, epigenetic memory of the donor tissue has been shown to influence iPSC differentiation potential, causing a differentiation bias towards lineages related to the donor cells. In addition, transplantation of differentiated iPSC into mice demonstrates a propensity of these cells to form teratomas, by unknown mechanisms that do not involve transgene reactivation. These differences between iPSC and hESC may be problematic for the clinical application of iPSC technologies. Here we report that human iPSC, compared with hESC, exhibit a propensity to revert to a pluripotent phenotype after spontaneous or directed differentiation. Initially, we used our published method to obtain sub-fractions of iPSC and hESC by fluorescence activated cell sorting (FACS), based on expression of the cell surface markers TG30 (CD9) and GCTM-2. Our previous findings demonstrated that the TG30Hi-GCTM-2Hi (P7) sub-fraction contains the highest proportion of pluripotent cells, whereas the TG30Neg-GCTM-2Neg (P4) sub-fraction contains a very high proportion of differentiated cell types. Continuing with the characterization of P4 and P7 cell sub-fractions, we found that spontaneously differentiated cells (TG30Neg-GCTM-2Neg) collected and cultured post-FACS from multiple human iPSC lines were able to re-acquire immunoreactivity to TG30 and GCTM-2, formed stem cell-like colonies and robustly formed both embryoid bodies and teratomas. In contrast, spontaneously differentiated TG30Neg-GCTM-2Neg hESC, processed in an identical fashion, did not re-acquire immunoreactivity to TG30 and GCTM-2, formed negligible stem cell-like colonies and did not form teratomas. We also found that this reversion was stable and reproducible even in late-passage (>40 weekly passages) iPSC lines, without signs of attenuation. Finally, the propensity to revert to a pluripotent phenotype was also seen in human iPS cell lines following a 28 day neural differentiation protocol and FACS for polysialyated NCAM positive cells. We are currently investigating the mechanism(s) responsible for this iPSC phenomenon in multiple human iPS cell lines derived from various somatic cell sources and made using both genome integrative and non-integrative technologies.

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Poster Board Number: 3094

INVESTIGATION FOR DIFFERENTIAITON-RESISTANCE IN HUMAN INDUCED PLURIPOTENT STEM CELLS

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Embryonic stem (ES) cells are capable of unlimited proliferation and differentiation into all cell lineages. Induced pluripotent stem (iPS) cells were established from somatic cells with introducing defind factors and have a similar character to ES cells. Although one of most expected use of the iPS cells is cell therapy, residual undifferentiated cells can develop teratomas after *in vivo* transplantation if not all cells are induced differentiation. Here, we uncovered abnormal iPS clones persisting undifferentiated state after induction of neural differentiation and investigated their difference from nomal ES and iPS cells. First, we found there were some differentiation-resistant human iPS cell clones when human ES and iPS cells cultured with inhibitor of BMP and TGFβ/Actin/Nodal signal formed neurospheres (SFEBq). Flowcytometry analysis showed 4 clones, including 1 cord blood-derived clone, 1 transgene-free clone and 1 clone established without c-MYC, remained OCT3/4 expressing cells, whereas the other clones were highly differentiated into PSA-NCAM+ neural cells after 14 days SFEBq differentiation. Next, we performed gene expression and miRNA microarray analysis for comparing normal and abnormal clone groups. However, no significant candidate gene was shown. We investigate the possibility that only a part of all population of abnormal clone might represent differentiation-resistance. Subclones established from single cells of each abnormal clone were performed with SFEBq, and some of them were well-differentiated without remaining TRA-1-60+ undifferentiated cells. Moreover, we isolated TRA-1-60+ cells from abnormal clone-derived neurospheres (NSTPs). Using such subclones and NSTPs, more effective comparable analysis was performed with microarray. As a result, we found 6 candidate genes significantly different expressing between normal and abnormal subclones. For the future, the functional relation between abnormality and these 6 genes would be studied.

Poster Board Number: 3096

COMPARISON OF GENETICALLY IDENTICAL HUMAN EMBRYONIC STEM CELL (HESC) AND INDUCED PLURIPOTENT STEM (HIPS) CELL LINES

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Since the discovery of methods to derive human induced pluripotent stem cells (hiPSCs) from somatic cells, numerous studies were published. hiPSCs provide a valuable tool for understanding disease mechanisms, model for human development, and for drug discovery. iPS cells share similar features with hESCs and for many aspects are identical to them. However, the comparison of hESC and iPSCs is obstructed by their different genetic backgrounds. The aim of our study was to establish and compare genetically identical hESCs and iPS cell lines. In our study we established hESC line from hESM05 line with Dox-inducible lentiviral insertion of 5 transcription factors (Oct3/4, Sox2, KLF4, c-Myc, Nanog). This line designated as hESM05neo2 was tested for pluripotency and further differentiated into pure population of neural cells. Then, Dox-system was activated in hESM05neo2-derived neural cells by doxycycline, and first iPS clones were picked up at day 16 after induction. Reprogramming efficiency was about 10%, iPS clones were characterized by RT-PCR analysis, karyotype analysis and immunocytochemistry. The clones were able to differentiate into cells of three germ layers. We demonstrate the results of transriptome and methylome comparison of neural-derived hiPS clones, hESC clone, and hESC-derived neural cells.

Poster Board Number: 3098

DISSECTING THE CELLULAR PATHOGENESIS OF HERPES SIMPLEX ENCEPHALITIS IN NEURONS AND GLIA DERIVED FROM PATIENT-SPECIFIC UNC-93B-DEFICIENT IPS CELLS.

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Childhood Herpes Simplex Encephalitis (HSE) results, in at least some cases, from inborn errors of UNC-93B-dependent, TLR3 immunity. In primary and SV40-transformed dermal fibroblasts from the patients with molecularly defined HSE, impaired TLR3-dependent induction of IFN-α/β and -λ results in enhanced HSV-1 replication and increased cell death. To test the hypothesis that a similar process operates in the Central Nervous System (CNS), we derived induced Pluripotent Stem Cells (iPSCs) from UNC-93B-deficient and normal human primary dermal fibroblasts. These iPSCs were successfully differentiated into purified CNS populations of Neural Stem Cells (NSCs), neurons, oligodendrocytes and astrocytes. In all UNC-93B-deficient cell types, there was impaired induction of IFN-β and/or -λ upon TLR3 stimulation. Moreover, consistent with impaired IFN-β or -λ induction upon HSV-1 infection, UNC-93B-deficient neurons and oligodendrocytes, but not astrocytes, showed higher vulnerability to HSV-1 infection as compared to healthy control cells. This is the first study to provide a tissue-specific molecular and cellular basis for childhood HSE, a devastating viral disease, by demonstrating impaired TLR3-dependent IFN-α/β and -λ immunity to HSV-1 in UNC-93B-deficient CNS neurons and oligodendrocytes.

Poster Board Number: 3100

GENERATION OF HUMAN DISEASE-SPECIFIC INDUCED PLURIPOTENT STEM CELLS

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The epigenome of differentiated somatic cells can be reprogrammed to a pluripotent state by overexpression of defined transcription factors. This new approach greatly simplifies the generation of pluripotent stem cells, bypassing many technical and ethical hurdles. The derivation of pluripotent stem cells from somatic tissues has provided researchers with a renewable source of patient-specific stem cells. Potentially, it offers a new paradigm for modeling human disease and for individualizing drug testing. Here we reported that a series of disease-specific induced pluripotent stem (iPS) cells were generated by transducing reprogramming factors into human dermal fibroblasts from disease patients isolated via informed consent, which include diabetes type 1, amyotrophic lateral sclerosis (ALS), metachromatic leukodystrophy (MLD), and Krabbe disease.

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Poster Board Number: 3102

GENERATION OF DISEASE-SPECIFIC INDUCED PLURIPOTENT STEM CELLS AND DIFFERENTIATION INTO DISEASE-SPECIFIC CELL TYPES

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Induced pluripotent stem cells (iPSCs) derived from patients possess the same genetic information as patients. These cells would provide critical evidences for determining mechanisms of disease pathogenesis. Here, we report that iPSCs are generated using adult fibroblasts taken from patients with diverse disease backgrounds including Alzheimer's disease (AD), Adenoleudodystrophy (ALD), Parkinson's disease (PD), Duchenne type muscular dystrophy (DMD), Juvenile-onset, and type I diabetes mellitus (JDM). These skin fibroblasts were transduced with retroviral vectors containing the 4 reprogramming genes (OCT4, SOX2, KLF4, and c-MYC). After 4 weeks of viral transduction, ES-like colonies were obtained, respectively. These cells were successfully expanded by mechanical passaging. All of them showed intense alkaline phosphatase activity, exhibited characteristics of human embryonic stem cell (hESC) morphology, expressed pluripotent markers including OCT4, NANOG, SOX2, SSEA4, TRA1-60, and TRA1-81. DNA fingerprinting analyses for microsatellite markers verified that iPSC lines were indeed derived from their parental skin fibroblasts. RT-PCR revealed that the endogenous pluripotency-associated genes, OCT4, SOX2, NANOG, hTERT, REX1, and GDF3, were robustly expressed in the iPSCs as in hESCs, while the expression of retrovirus-delivered transgenes was barely detectable in the iPSC lines. Furthermore, the iPSC lines were able to spontaneously differentiate into the three embryonic germ layers (ectoderm, endoderm, and mesoderm). Taken together, our results demonstrated that disease-specific iPSCs closely resembled hESCs and retained the capability to differentiate into all cell types of human body. With the patient-specific iPSCs, we induced differentiation and obtained disease related cell types such as oligodendrocytes and neurons. We are now studying for discovering disease mechanisms and screening new drug compounds with the differentiated cells. Our results will be announced on the poster. This research was supported by grants from the Stem Cell Research Center of the 21th Century Frontier Research Program funded by the Ministry of Education, Science and Technology, Republic of Korea (SC1110) and from Korean Health Technology R&D Project, Ministry for Health, Welfare & Family Affairs (A100694).

Poster Board Number: 3104

MODELING VASCULAR LESIONS ASSOCIATED WITH AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE USING PATIENT-SPECIFIC IPSCS

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Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent, potentially lethal, monogenic disorder, characterized by the development of multiple renal cysts and various extrarenal manifestations. Cardiovascular complications are the main cause of death in patients with ADPKD, including hypertension, intracranial aneurysms and dolichoectasias, thoracic aortic and cervicocephalic artery dissections, coronary artery aneurysms and valvular heart abnormalities. The pathogenesis of cardiovascular lesions as well as renal cyst formation remains largely unknown, and no therapeutic strategies have been established. Here we report the derivation of induced pluripotent stem cells (iPSCs) from skin fibroblast samples from seven patients with ADPKD. These cells expanded robustly in culture and differentiated into vascular endothelia and mural cells *in vitro*. Using this differentiation system, we have identified several molecules whose expression levels were upregulated or downregulated in vascular cells differentiated from ADPKD-iPSCs as compared to those from normal Japanese iPSCs. These results suggest that disease modeling using patient-specific iPSCs can be used for studying the mechanisms of vascular complications associated with ADPKD.

Poster Board Number: 3106

INTRODUCTION OF THREE NOVEL IPS DISEASE MODELS, AND A REPOSITORY FOR THE PRESERVATION AND DISTRIBUTION OF IPS LINES

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The ability to reprogram a somatic cell to the induced Pluripotent Stem Cell (iPS) state enables the creation of *in vitro* models of human disease. The impact these models will have on the discovery and testing of novel therapies will be largely determined by two parameters: the breadth and genetic diversity of the models created; and the accessibility of those models to medical researchers around the world. To address the issue of iPS cell diversity, we strived to achieve the establishment of an efficient method of iPS cell reprogramming that could be routinely used to generate iPS lines from tissue samples harboring mutations of developmental disease. We report here the creation and characterization of three novel iPS lines reprogrammed from fibroblasts donated by patients with osteogenesis imperfecta, Down's syndrome, and cystic fibrosis. All lines, and a wild type control line, were created by transient expression of the reprogramming genes Oct4, Sox2, Klf4 and Myc and harbor no gene insertion events or residual expression of reprogramming genes. All lines are characterized for expression of pluripotency markers, ability to differentiate, and presence of disease-specific genetic mutations. To address the issue of accessibility, we created the

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ATCC® iPS Cell Repository. This Repository continues the ATCC mission to make relevant models of human disease accessible to medical researchers worldwide. These and other iPS lines, the origin fibroblasts, and fibroblasts from relatives and control lines will be available from the Repository through the ATCC catalog.

Poster Board Number: 3108

GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS FROM PATIENTS WITH EARLY-ONSET CARDIOVASCULAR DISEASE

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Myocardial infarction is a leading cause of mortality and morbidity worldwide. In addition to traditional risk factors, several studies have confirmed that a family history of cardiovascular disease (CVD) is an independent risk factor. Myocardial infarctions in younger individuals have been associated with substantially greater heritability. Thus, early-onset myocardial infarction with confirmed family history is a promising phenotype for mapping genetic risk factors for CVD. At the Academic Medical Centre (AMC) in Amsterdam we have identified pedigrees with several cases of premature atherosclerosis (PAS). It is tempting to speculate that novel, high-penetrance mutation segregates in these pedigrees. We aim to identify these sequence variations and determine how these may result in CVD by studying the effect on cell phenotype by using human induced pluripotent stem cells (hiPSCs). We isolated human fibroblast cell lines from skin biopsy of PAS patients. The affected fibroblasts were reprogrammed with four lentiviral vectors encoding for the pluripotent transcription factors c-Myc, Sox-2, Klf 4 and Oct-4. Six weeks later, the first fully reprogrammed colonies were detected. The PAS-hiPSCs were morphologically analyzed and evaluated for the positivity for the pluripotent marker. Furthermore the differentiation potential into the three germ layers *in vitro* was analyzed. We successfully reprogrammed hiPSCs from four patients. The characterization revealed their efficient functionality and their use for further applications. We are focused to differentiation the PAS-hiPSCs into different lineages mainly part of the vascular system (i. e. endothelial cells, smooth muscle cells) in order to study the disease mechanisms.

Poster Board Number: 3110

IPS CELLS: A NOVEL CELLULAR SOURCE FOR MUCOPOLYSACCHARIDOSIS TYPE I

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MPS I is an autosomal recessive disease, belonging to lysosomal storage disorders and it is caused by mutations in the IDUA gene, resulting in the deficiency of α-L-iduronidase enzyme. The intra-cellular accumulation of its substrates in all body tissues causes progressive deterioration and abnormal function of multiple organs. Recently, a new kind of pluripotent stem cells, named induced Pluripotent Stem cells (iPS), were isolated through the reprogramming of adult somatic cells with the over-expression of four defined transcription factors. Once established, iPS cells are transcriptionally and epigenetically similar to embryonic stem (ES) cells and they normally maintain the developmental potential to differentiate into derivatives of all the three primary germ layers. We aim to use iPS cells as a tool to elucidate MPS-I mechanisms, with a particular focus on the skeletal and neural abnormalities. We isolated human fibroblast cell lines from skin biopsy of MPS-I patients. Subsequently, we reprogrammed the affected fibroblasts with four lentiviral vectors encoding for the pluripotent factors c-Myc, Sox2, Klf 4 and Oct-4. Four weeks later, the first fully reprogrammed colonies arose in the culture; we selected and then characterized few iPS cell lines. To fully characterize MPS-I iPS, the cells were morphologically analyzed and the isolation efficiency was determined. We then evaluated the positivity for the pluripotent markers and the content of endogenous and exogenous expression of the transduced genes through real time PCR. We also assessed viral integration copy number. Furthermore, we tested their differentiation potential into the three germ layer *in vitro* and finally, we injected iPS into immunodeficient mice for teratoma induction. We successfully isolated several iPS cell lines from two MPS-I patients; the complete characterization revealed their efficient functionality and their possible use for further applications. Indeed, our ongoing studies are focused to assess the differentiation potential of MPS-I iPS cell lines into different lineages mainly affected by the disease (i.e. bone, cartilage and neurons).

Poster Board Number: 3112

MODELING HUMAN PROTEIN-INDUCED DISEASES LINKED TO DESMIN-RELATED CARDIOMYOPATHY WITH INDUCED PLURIPOTENT STEM CELLS

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Chronic conditions such as heart failure and Alzheimer's are characterized by protein misfolding states for which the underlying mechanism(s) and effective therapies remain poorly defined and elusive goals, respectively. Direct reprogramming of somatic cells into induced pluripotent stem cells (iPSC) using defined transcription factors provides a robust platform for studies of pathophysiology, enables the development of patient-specific model systems, and opens a gateway for screening novel disease-specific therapies for personalized medicine. Missense mutation of the small MW heat shock protein CryAB causes an inheritable multisystem disorder characterized by cataracts, proximal muscle weakness, cardiomyopathy, and sudden cardiac death in humans. Our laboratory has challenged the existing paradigm of oxidative stress by demonstrating that mouse hearts exhibiting protein-folding cardiomyopathy found in humans are under 'reductive stress' from an over-active antioxidative system. Decreasing the function of glucose-6-phosphate dehydrogenase (G6PD), which generates the reductant NADPH, "cures" the disease in mice by ameliorating reductive stress, aggresome formation, hypertrophy, heart failure and death. To further understand the molecular mechanisms, we have harvested dermal fibroblasts from transgenic mice harboring wild type and transgenic R120G CryAB under the control of alpha-MHC promoter for generation of iPSC containing both human wild type and R120G mutant CryAB proteins. From several clones after four-factor reprogramming (OCT4, SOX2, KLF4, and C-MYC) that showed typical size and morphology of pluripotent stem cells, we have begun initial characterization after differentiation of iPSCs into cardiomyocytes containing protein aggregates. In Preliminary Studies, we will report on our progress to define their metabolic profiles using GC-MS and LC-MS. In addition, we will determine whether distinct molecular signatures at the post-transcriptional levels using Micro-RNA arrays accompany these metabolic perturbations. In summary, our study will illustrate whether iPSC technology will be robust platform that recapitulates certain cellular, molecular and biochemical properties of protein-induced diseases found in humans.

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Poster Board Number: 3114

EFFECTIVE DIFFERENTIATION INTO CARDIOMYOCYTE FROM XENO-FREE CONDITION CULTURED HUMAN IPS CELLS

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Background: Cell therapy for myocardial repair after myocardial infarction is a promising strategy. Human Induced pluripotent stem (hiPS) cell-derived cardiomyocytes hold significant promise for clinical applications. However, in most of protocols for not only maintaining undifferentiated state but also differentiation induction, the animal derived products are used such as feeder cells and medium containing FBS or serum replacement, which might adversely affect in clinical situation. We believe it is better to use as much of xeno-free condition protocols as possible. In this study, we investigated whether hiPS cells in xeno-free conditions can differentiated into cardiomyocyte effectively. Methods & Results: hiPS cells were maintained both on synthemax surface in xeno-free medium and on MEF feeder cells in defined medium including 20%KSR. For differentiation into cardiomyocytes, hiPS cells were floating-cultured and induced by WNT signaling molecules. The efficiency of cardiac differentiation was determined by the number of beating conies, immunostaining and RT-PCR. Using hiPS cells in xeno-free conditions, the number of beating colonies in WNT signaling molecules treated group was more than 30-fold higher than that in WNT signaling molecules untreated group. Moreover, the number of beating colonies from hiPS cells in xeno-free conditions was higher than that from hiPS cells in MEF feeder cells-conditions (1.37±0.11-fold; $p<0.01$). Conclusion: These data demonstrate that WNT signaling molecules can effectively differentiated into cardiomyocyte from hiPS cells in xeno-free conditions and suggest that hiPS cells in xeno-free conditions have more potential for cardiac differentiation than in MEF feeder cells-conditions. This protocol from maintenance of hiPS cells to cardiac differentiation can be able to scale up easily and it might solve some questions in regard to clinical application of hiPS cells for cell therapy of myocardial repair.

Poster Board Number: 3116

TRACKING THE GENOMIC STABILITY OF HUMAN INDUCED PLURIPOTENT STEM CELLS BY BOTH KARYOTYPING AND HIGH-RESOLUTION ARRAY-BASED COMPARATIVE GENOMIC HYBRIDIZATION

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Genomic stability of human induced pluripotent stem cells (hiPSCs) is essential to safeguard their value for research and therapeutic applications. Here we monitored the genomic stability of 32 hiPSC lines by G-banded karyotyping and high-resolution array-based comparative genomic hybridization (aCGH) analysis. Few aneuploidies were detected in hiPSCs by karyotyping, following extended periods in culture. Using aCGH, we identified unique copy number variation (CNV) signatures for hiPSC lines derived from specific sources of parental fibroblasts, including CNVs shared between high and low passages and CNVs acquired specifically by hiPSCs during culture. Recurrent CNVs at 1q31.3 and 17q21.1 were shared by > 25% of hiPSCs. Furthermore, the loss of 8q24.3 was unique to hiPSCs. Additional recurring CNVs at 2p11.2 and 20q11.21 were acquired by both hESCs and hiPSCs. Our results suggest that both karyotyping and aCGH are required for the analysis of the genomic stability of hiPSC lines.

Poster Board Number: 3118

MAINTENANCE OF HUMAN IPS CELLS ON DENDRIMER SURFACE WITH D-GLUCOSE-DISPLAYING

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Introduction: The culture technique for maintenance of human induced pluripotent stem (iPS) cells is not sophisticated owing to extremely high cost and tedious labor, leading to developing issues for the culture environment (ex. feeder-free medium and artificial substrate to remove feeder cells and nature materials, respectively) and the operation (automation system). In the present study, we develop a novel artificial substrate for maintaining undifferentiated state of iPS cells by using the dendrimer surface with D-glucose-displaying. Materials and Methods: The cultures of iPS cells (clone 201B7 provided from RIKEN Bioresource Center in Japan) were conducted in medium of ReproStem (ReproCELL, Japan) on the substrates (conventional tissue culture plastic surface with gelatin coat and the dendrimer surface with d-glucose-displaying) with feeder layer of Mitomycin C-treated SNL76/7 cells (ECACC, UK). Here, the dendrimer surface was prepared according to the procedure as described elsewhere. To evaluate cell shape and function, immunostaining was performed using the standard protocol. Results and Discussion: The dendrimer surface was applied to prepare the feeder layer with feeder cells. The feeder cells exhibited the active migration with periodic change in cell morphology (round- and stretch-shapes), compared with that on gelatin-coated surface, suggesting the high potency of substrate for the maintenance of iPS cells due to the enrichment of extracellular matrix on the surface. The maintenance cultures were performed on the dendrimer surface and gelatin-coated surface and the undifferentiated state of iPS cells were estimated. The iPS cells on the dendrimer surface made packed colony with its defined edge. Analyses of alkaline phosphatase activity and immunofluorescence staining (OCT4, Nanog and SSEA-4) revealed that iPS cells in the colony on the dendrimer surface at 5 days maintained the characteristics of undifferentiated state, similarly to those on a gelatin-coated surface. Thus, the dendrimer surface is suggested to become an alternative substrate for iPS cells due to the activation of feeder cells.

Poster Board Number: 3120

REGULATED ECTOPIC EXPRESSION OF THE PLURIPOTENCY FACTORS SOX-2 AND OCT-4: A NOVEL APPROACH TO INSTALL REPROGRAMMING FACTORS IN HUMAN CELLS

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Transcription-factor-based reprogramming triggers dedifferentiation of adult (stem) cells back to an embryonic state. The obtained cell entities are called induced pluripotent stem cells (iPSC). They are able to generate all tissues across the three germ layers. Although the benefits of these cells are obvious, they remain far from clinical application. One major obstacle is the low efficiency of transcription-factor-based reprogramming. Beside the fact that reprogramming efficiency is strongly dependent on the respective cell type, it has been shown that minimal fluctuations of pluripotency factor concentration can induce cell fate decisions of embryonic stem cells. This

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led us to the hypothesis that defined levels of pluripotency factor expression might favor reprogramming in terminally differentiated cells as well as in adult stem cells. In order to check this hypothesis, we developed a method to overexpress defined quantities of the crucial pluripotency factors Sox-2 and Oct-4 and to track their intranuclear activity over the entire time period of the reprogramming process. In detail, the regulated gene expression is mediated by a non-viral plasmid vector system which has recently been developed in our laboratory (p2in1-Sox-2-IRES-Oct-4). It harbors a doxycycline-inducible promoter, regulating the expression of both a transactivator and the reprogramming factors Sox-2 and Oct-4. This vector was shown to promote adjustable gene expression in close correlation to the corresponding inductor concentrations. With the aim of detecting the functional expression of the reprogramming factors, we established an activity-based reporter-gene assay, capable of quantifying the functional levels of Sox-2 and Oct-4 in living cells using a Sox-2-Oct-4-dependent reporter gene plasmid (pN3-OSR). Due to secretion of the reporter protein (Gaussia luciferase), the intranuclear activity of the reprogramming factors can be quantified in the supernatant. This technique circumvents cell lysis and therefore allows continuous analysis of reprogramming factor activity. In summary, the two vector systems provide a powerful tool for a fine-tuned ectopic expression of the reprogramming factors Sox-2 and Oct-4 in combination with a simultaneous quantification of their intracellular activities during the entire reprogramming period.

Poster Board Number: 3122

GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS UNDER FEEDER AND SERUM FREE DEFINED CONDITIONS

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The successful establishment of human induced pluripotent stem cells (hiPSCs) has increased the possible applications of stem cell research in biology and medicine. However, one of the major obstacles to uses for hiPSCs is the risk of contamination from undefined pathogens in conventional culture conditions that use serum replacement and mouse embryonic fibroblasts as feeder cells. Here we report a simple method for generating hiPSCs under feeder- and serum-free defined culture conditions that we developed previously for human embryonic stem cells. The defined culture condition comprises a basal medium with a minimal number of defined components including five highly purified proteins and fibronectin as a substrate. We successfully generated hiPSCs using adult dermal fibroblast under the defined culture conditions from the reprogramming step. For a long term culture, the generated cells also had the property of self renewal and pluripotency, they carried a normal karyotype. Moreover, levels of nonhuman N-glycolylneuraminic acid (Neu5Gc), which is a xenoantigenic indicator of pathogen contamination in human iPS cell cultures, were markedly decreased in the generated hiPSCs. This study suggested that generation or adaption culturing under defined culture conditions can eliminate the risk posed by undefined pathogens. This success in generating hiPSCs using adult fibroblast would be beneficial for clinical application.

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HUMAN INDUCED PLURIPOTENT STEM CELLS FROM AN AUTISTIC INDIVIDUAL WITH MICRODELETION OF PTCHD1

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The study of neuropsychiatric diseases and the development of effective treatments have been limited by a lack of appropriate models. Induced pluripotent stem (iPS) cells represent a potentially limitless supply of patient-specific cells for the study of neuropsychiatric disorders. Here we describe the generation of iPS cells derived from an autistic male with an X-linked genomic copy number variation (CNV) that encompasses the promoter and first exon of the patched domain containing 1 (PTCHD1) gene, which encodes a patched homologue that has been implicated in regulation of the hedgehog signaling pathway. We also derived iPS cells from the proband's unaffected mother, who encodes both the wild type PTCHD1 allele and the CNV inherited by the proband. Skin fibroblasts were reprogrammed by retrovirus-mediated expression of OCT4, SOX2, KLF4, and CMYC. Successfully reprogrammed cells exhibited retroviral silencing, activation of endogenous reprogramming factors, and expression of pluripotency-associated genes including REX1, DNMT3b, and ABCG2. iPS cells derived from the proband's mother exhibited clonal, line-specific X chromosome inactivation. This has resulted in the generation of isogenic iPS cell lines that differ only with regards to their X chromosome inactivation status - and therefore - expression of the autism-associated PTCHD1 locus. Finally, we found that PTCHD1 is expressed in human embryonic stem cell-derived glutamatergic forebrain-like neurons, a cell type whose dysfunction has been implicated in the development of autism. Our future experiments will be focused upon directed-differentiation of proband and isogenic "control" and "mutant" maternal lines into (i) forebrain-like glutamatergic neurons and (ii) cerebellar granule cell-like neurons for characterization of gene expression and synapse formation, with the goal of identifying neuronal phenotypes that may underlie the development of autism. The cells described herein represent a potential model system for the elucidation of the molecular and cellular underpinnings of autism, and for understanding the role of PTCHD1 and the hedgehog signaling pathway in neuronal function and dysfunction.

Poster Board Number: 3126

FRAGILE X SUBJECT-DERIVED IPS CELLS AS A SOURCE FOR HUMAN NEURONS WITH CLINICALLY-RELEVANT FMR1 MUTATIONS

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Fragile X Syndrome (FXS) is an inherited neurodevelopmental disorder that causes mental retardation and autistic features in 1/5000 births, from every racial and socio-economic group. FXS is caused by expansions in the number of CGG repeats (>200) in the 5' untranslated region (UTR) of the FMR1 gene, which results in gene silencing and loss of the FMR protein (FMRP) - an RNA binding protein known to bind to and regulate the translation of a variety of mRNA species. This condition exhibits variable penetrance due to individual differences in CGG repeat length, methylation patterns of the FMR1 promoter and mosaicism - the locus is carried on the X chromosome. Although the Fmr1 KO mouse model displays dendritic spine abnormali-

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ties that are similar to those in FXS subjects, this animal model does not address the variable penetrance exhibited in humans as those mice are completely deficient for FMRP. We are developing an *in vitro* system to study the effects of clinically-relevant FMR1 mutations on synaptogenesis in large pyramidal neurons. Using primary fibroblast lines (12 lines) obtained from FXS subject skin biopsies, we have generated induced-pluripotent stem (FXS-iPS) cells using a 4-factor virus that forces expression of OCT-4, SOX-2, KLF-4 and c-MYC. The clones were grown 4 weeks before colonies with hESC-like morphologies were picked to generate FXS-iPS cell lines. The lines were expanded and characterized for the expression of SSEA-3/4, Alkaline phosphatase and OCT-4 expression. To produce the large pyramidal neurons required for this project, we developed a differentiation protocol using H9 human embryonic stem cells (hESC). The mixed neuron cultures obtained included large neurons with mature dendritic spines that stained for glutamatergic synapse markers. We are currently characterizing synapses and dendritic spine formation in FXS-iPS derived neurons, to determine if there are correlations between the gene methylation patterns, and dendritic spine dynamics. This approach will provide a disease-in-a-dish model to test the effects of new drugs on spiny neurons that carry clinically-relevant FMR1 mutations. Funding provided by FRAXA foundation and the California Institute for Regenerative Medicine.

Poster Board Number: 3128

BANKING HUMAN IPS CELLS GENERATED FROM ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS

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Induced pluripotent stem (iPS) cells were originally derived by reprogramming of human dermal fibroblasts through ectopic expression of pluripotency-associated transcription factors. A limitation to the use of dermal fibroblasts as the starting cell type for reprogramming is that it usually takes weeks to expand cells from a single biopsy, and the efficiency of the process is very low. In contrast to human dermal fibroblasts or terminally differentiated cell sources, a large number of adipose-derived mesenchymal stem cells (Ad-MSCs) can be easily obtained from human fat tissue, without the time-consuming steps of cell expansion. The successful reprogramming of Ad-MSCs into iPS cells may provide an ideal autologous source of cells for generating individual-specific iPS cells. Patient-specific iPS cells will facilitate our basic understanding of specific disease mechanisms. More importantly, the generation of patient-specific and disease-specific iPS cells has the potential to greatly impact regenerative medicine, and facilitate drug and vaccine development. The aim of this study is to generate a bank of human iPS derived from Ad-MSCs. The starting point is the derivation of Ad-MSCs from human adipose tissue obtained from liposuction or discarded fat from surgical procedures. The Ad-MSCs were then used to generate iPS cells by ectopic expression of "Yamanaka's cocktail" containing OCT4, SOX2, KLF4 and c-MYC. The success rate is very high compare to iPS cells generated from human dermal fibroblasts, almost all Ad-MSCs cell lines can be reprogrammed into iPS cells. These cells present the same characteristics as skin fibroblasts-derived iPS cells and human ES cells in their morphology, gene expression profile and differentiation capacities. We are now optimizing this approach and making it more clinically useful by adopting an integration-free method to deliver the reprogramming factors.

Poster Board Number: 3130

GLOBAL ANALYSIS OF PARENTAL IMPRINTING IN HUMAN PARTHENOGENETIC INDUCED PLURIPOTENT STEM CELLS

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Parental imprinting is a phenomenon affecting human development and disease. In order to analyze the role of parental imprinting in human embryogenesis, we generated parthenogenetic human induced pluripotent stem cells (PgHiPSCs), by genetic manipulation of parthenogenetic ovarian teratomas. PgHiPSCs show typical characteristics of pluripotent cells and have a homozygote diploid karyotype. Global gene expression analysis of the parthenogenetic cell lines correctly identified most of the known paternally expressed genes. In addition, genome-wide DNA methylation analysis of the parthenogenetic cells demonstrated differential methylation in imprinted loci. We could demonstrate that a variant of the U5 RNA component of the spliceosome (U5d), which is highly enriched in pluripotent stem cells, is a novel imprinted gene, as it is down regulated in the parthenogenetic cells, and expressed in a monoallelic fashion in the wild type cells. Introduction of the U5d gene to the parthenogenetic cells corrected the expression of several of the aberrantly expressed genes. Our analysis also uncovered multiple miRNAs as novel imprinted clustered transcripts, and studied their putative targets. In order to study the consequences of parthenogenesis on human development, we differentiated the PgHiPSCs *in vitro* and *in vivo*, demonstrating marked effects on the differentiation of the extra-embryonic trophectoderm, and of the embryonic liver and muscle tissues. This analysis provides a global view on human imprinting, and suggests a significant involvement of distinct regulatory imprinted small RNAs and their targets in human development.

Poster Board Number: 3132

SMALL MOLECULES IN INDUCING PLURIPOTENCY OF HUMAN FIBROBLASTS THROUGH CHEMICAL GENOMICS

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Human induced pluripotent stem (iPS) cells offer great potential for cell-based therapies, *in vitro* disease modeling, drug discovery and toxicology. To realize the full potential of iPS cell technology, two major hurdles must be addressed: the reliance on viral vectors to deliver reprogramming factors, and the inefficiency of the process when applied to human cells. Small molecules could potentially replace the virally delivered reprogramming factor(s) or enhance reprogramming efficiency. Indeed, iPSCs have recently been generated from primary somatic cells with only one gene OCT4 and chemical compounds, although the efficiency leaves much to be desired. We have established a screening assay for reprogramming, in which stable human fibroblast cell lines were established to express 2 sets of 3 factors (Sox2, Klf4 and c-Myc, and Oct4, Sox2 and Klf4) under the control of Tetracycline (Tet) and hence can be used to screen for small molecules that function in the place of the missing factor when other 3 factors are induced in the presence of Tet. With this assay system, we are carrying out 2 parallel screens of 100,000 compounds for their ability for reprogramming. A few dozens of primary hits have been identified, and further confirmatory and secondary assays measuring molecular markers of pluripotency (e.g., AP, Tra-1-60, SSEA4, NANOG) are being employed to validate these hits. Mechanistic studies of the confirmed hits will likely identify novel genes / pathways involved in reprogramming and shed light on the mechanisms of cellular reprogramming in human cells.

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THE AUTOMATED CELL PROCESSING OF HUMAN INDUCED PLURIPOTENT STEM CELLS (HIPS CELLS) IN LARGE AMOUNT FOR INDUSTRIAL USE.

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Kawasaki Heavy Industries, LTD. (KHI), National Institute of Advanced Industrial Science and Technology(AIST), and National Center for Child Health and Development(NCH) have succeeded in culturing human induced pluripotent stem cells (hiPS) automatically for about 4 months continuously by using the automated cell processing machine which KHI has developed. HiPS cells can be established from mature somatic cells, so it can clear the ethical problems easily compared to hES cells, which require to destroy a fertilized ovum to be obtained. Therefore, hiPS cells are expected to become common in the field of regenerative medicines or drug discovery. To accelerate the industrial use of hiPS cells, it is necessary to supply cells in large amount stably. The automated cell processing machine which can deal with hiPS cells can be the best solutions. It is important to keep the cells undifferentiated (pluripotency) during the culture for the further use. However, processing hiPS cells is difficult compared to established cell lines, and the special technique of the skilled technician is required. The KHI's cell processing machine can duplicate the manual handling of the technicians by using clean robot and the other special equipments to automate the process of cell culture. Since they can move in programmed way, they will not make careless mistakes, and they can repeat the same movement completely with high reproducibility. On the other hand, culture parameters can be modified by users for every operation according to the condition of cultured cells. The clean robot carry out culture operations inside the chassis where the clean class is kept virtually 0, so there is no risk to contaminate. The automated processing of hiPS cells have tried by applying these features of the machine. In this trial, the hiPS cell line derived from MRC5 cell line (established by NCH) was cultured automatically using commercially available dishes. During the trial, passage cultures were executed once a week and medium replacement were executed everyday automatically for about 4 months. The cultured hiPS cells were observed by phase-contrast micrograph to check the morphology of cultured hiPS colonies. As the marker of undifferentiated hiPS cells, expression of Oct3/4, Nanog, Sox2, SSEA4 and Tra-1-60 were verified by immunostaining. Also expression of alkaline phosphatase was confirmed to check all colonies in the dishes. As a result, more than 90% of colonies showed positive reaction by ALP staining. In addition, the cultured hiPS cells were transplanted to immunodeficient mouse to check the pluriopotency. After 2 months, tumor formation was confirmed and tissues from endoderm, mesoderm, and ectoderm were observed by HE staining. The karyotype of cultured hiPS cells were also tested and verified that they were kept normal after 15 passages. For the next step, culture of more number of hiPS cell lines, automated colony screening by using the image processing technology in the machine will be tested. This machine will be able to apply for the regenerative medicine in the future because KHI has already developed another version of cell processing machine which can adapt GMP standard. This machine can contribute the industrial use of hiPS cells significantly.

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HETEROGENEITY IN EARLY HUMAN INDUCED PLURIPOTENT STEM CELL COLONIES

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Somatic cells can be reprogrammed to pluripotent state by ectopic expression of defined transcription factors. The acquisition of a pluripotent phenotype is a stochastic process with intermediate cell states resulting in only part of the cells reaching pluripotency during the induction. To distinguish partially from fully reprogrammed cells, several features associated with pluripotent cells can be monitored live in early forming iPS cells. These properties include for example typical morphology, silencing of retrovirally delivered transgenic factors and expression of specific cell surface antigens. To decipher how the pluripotent state is acquired in human cells, we have monitored transgene silencing and surface marker expression by live imaging during the generation of iPS cells. Human foreskin fibroblasts were transduced with retroviruses encoding Oct4, Sox2, Klf4 and c-Myc coupled to EGFP with self cleaving 2A sequences. Cells were split on day 3 post transduction on either mitotically inactivated mouse embryonic feeder cells (MEF) or matrigel, and cultured in reprogramming medium since day 6. Cells were monitored for GFP and Tra-1-60 expression under fluorescent microscope or in an automated live cell imaging system (Cell-IQ ®). iPSC colonies were plated either on MEFs using conventional hES medium or on Matrigel using StemPro medium. Majority of the colonies turned GFP negative early on during reprogramming, indicating the silencing of transgenes. However, a subset of the forming colonies showed heterogeneous GFP positive cell populations within colonies that otherwise showed morphological properties and Tra-1-60 positivity typical to bona fide iPS colonies. Automated live cell monitoring revealed that some of the heterogeneously fluorescent colonies were formed by fusion of multiple smaller colonies originating from separate reprogramming events.GFP expression diminished significantly during the first passage, faster in cells cultured on Matrigel than on MEFs. Our results show that pluripotent reprogramming of human somatic cells can lead to heterogeneous cell populations measured by transgene silencing. Our data also proposes that culture conditions after colony formation might affect the outcome of the reprogramming process and quality of the iPS clones.

Poster Board Number: 3138

COMPARATIVE ANALYSIS OF THE ROLE OF USP44 IN HUMAN EMBRYONIC STEM CELLS, RETROVIRAL AND mRNA-DERIVED AMNIOTIC FLUID INDUCED PLURIPOTENT STEM CELLS

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Human amniotic fluid cells (AFCs) have gained increasing appreciation in the biomedical field over the last decade. Due to the presence of fetal stem cells within this heterogenic mixture of cells, which have multipotent capacities and possess putative immune privilege characteristics, primary human AFCs are believed to be valuable for conducting disease modeling, pharmaceutical and toxicological studies and even cell-based regenerative therapies in the future. Adding even greater value to AFCs, we and others demonstrated very recently that amniotic fluid cells enable fast and efficient induction of pluripotency, whereby senescence of primary human AFC cultures is bypassed and their differentiation potential enhanced. We extensively characterized several retrovirally generated human amniotic fluid-derived induced pluripotent stem cell (AFiPSC) lines and work with these established AFiPSC lines in different follow-up studies. We will present the current status

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of two of our ongoing AFiPSC-based projects: (I) We focus on the optimization of an improved non-viral, synthetic mRNA-based reprogramming technique. As our retroviral-derived AFiPSC lines harbour several integration events within their genomes with yet unknown effects on the transcriptome it is our goal to generate integration-free AFiPSC lines. We anticipate that the stem cell-like cells present in bulk primary AFC cultures facilitate the realization of this otherwise rather complex, inefficient mRNA reprogramming approach. This will give us the opportunity to compare the transcriptomes and differentiation potential of our retroviral and mRNA-derived induced pluripotent stem cell (iPSC) lines harbouring identical genetic background. (II) We aim to compare the downstream effects of knocking down a gene in the AFiPSC lines and embryonic stem cells (ESCs). For this purpose we chose to knock down the gene encoding the deubiquitinating enzyme USP44. This enzyme is a critical regulator of the spindle checkpoint during cell cycle. It has been shown to prevent premature activation of the anaphase promoting complex. Using an OCT4 ChIP-on-chip approach, we and others identified USP44 as a positively regulated OCT4 target gene in various human pluripotent cell lines. We also demonstrated that the USP44 proximal promoter harbours an evolutionary conserved OCT4 binding site and we were able to confirm OCT4 binding by ChIP-real-time-PCR. Yet, the exact role of USP44 in the maintenance of self-renewal and pluripotency is unknown. Therefore, utilizing our AFiPSC lines as well as the ESC lines H1 and H9, we will aim at deciphering its function. As a result of these studies we expect to be able to attain the following: (I) unveil similarities and differences between viral and *in vitro* mRNA-based reprogramming methods on the transcriptome, long-term stability and differentiation capabilities of iPSC lines derived from the same genetic background. (II) unravel the role of the pluripotency-associated gene USP44 on self-renewal and the maintenance of pluripotency in human ESCs and iPSCs.

Poster Board Number: 3140

REVEALING THE ONCOGENIC RISK OF HUMAN INDUCED PLURIPOTENT STEM CELLS USING *IN VITRO* CARTILAGE TISSUE ENGINEERING.

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Induced pluripotent stem cells (iPSCs) are attractive cells for regenerative medicine. However, iPSCs may have the risk of oncogenic transformation due to the use of proto-oncogenes in reprogramming, in addition to the use of viral vectors and random genomic integrations. Indeed, an incident of tumor formation was observed in mouse iPSC-derived chimeras. In this regard, chimera formation is a useful indicator for oncogenic potential in mouse iPSCs. Unfortunately, this risk remains unclear for human iPSCs, as a useful indicator has been lacking. What is needed is a useful indicator for evaluating the risk of oncogenic transformation in human iPSCs. Here, we demonstrate how we have discovered a new way to reveal the oncogenic risk of human iPSCs using *in vitro* cartilage tissue engineering. Following micro-mass chondrogenic differentiation, both human ESCs and iPSCs displayed similar cell morphology. Although cartilage specific histological staining is present in cartilage derived from both cell types after 6-8 weeks of differentiation culture, upon closer inspection we observed the presence of mucous glands or adenocarcinoma-like cysts within iPSC-derived cartilage based on morphology, lack of cartilage matrix, proteoglycan, and the expression of tumor markers, CEA and CA19-9. Non-cartilage specific genes, such as the pro-oncogenic/pluripotency c-Myc and Lin28B were also up-regulated in pro-oncogenic iPSC lines. The penetrance of this adenocarcinoma phenotype could be modulated down by the p53 agonist, Nutlin-3, or up by the antagonist α-pifithrin. Nutlin-3 suppresses tumor formation by activating the p53 pathway, which resulted in apoptosis or cell cycle arrest of cartilage differentiated iPSCs cultures. This adenocarcinoma-like phenotype was observed in two of three human iPSC lines, which we have studied thus far. These results indicate that certain human iPSCs may have pro-oncogenic/adenocarcinoma potential and reveal their risks following differentiation into cartilage. Considering the aforementioned observations, cartilage tissue engineering may be an excellent tool for screening human iPSCs *in vitro*, without the need for transplantation into animals. This system also shows the potential to identify the key regulators of oncogenic transformation of pro-oncogenic human iPSCs and to adapt similar approaches to the study of cancer stem cells. There are several variations between iPSC lines owing to genomic insertional mutagenesis. Since safety is a foremost issue in human iPSC application, cartilage tissue engineering may provide a useful safety evaluation approach.

Poster Board Number: 3142

DIRECT DIFFERENTIATION OF SUSPENDED SPHEROID FORM OF HIPSCS TO ENDODERM PROGENY

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Definitive Endoderm (DE) and particularly foregut is the primary origin of functional cells in parenchyma of liver and pancreas. Many studies have been conducted to produce hepatocytes and beta cells from pluripotent cells, however the acceptable functionality and adequate quantity of generated cells are two main challenging issues that have been remained undisclosed yet. Simulation of the specific niche of cells during development and application of appropriate growth factors are the basic considerations in direct differentiation protocols of stem cells into favorite mature cells. Static culture of cells in plates in two dimensional (2D) forms and their differentiation in experimental scale through expensive growth factors cannot product adequate cells for therapeutic application. Suspension culture of pluripotent cells in spheroid form has enabled researchers to handle the culture of enormous amount of stem cells in limited space and helped them to save their time and budget. The replacement of expensive growth factors with suitable chemicals will be the second solution for cell production in therapeutic scale. In this study we performed direct differentiation of human induced pluripotent stem cell lines in the suspended condition to DE in different groups. We demonstrated that lower concentrations of ActivinA (50ngr/ml) in correlation with small amounts of Rapamycin can stimulate the expression of specific genes of DE such as SOX17 and FOXA2. This increase in gene expression in some groups is even higher than control group with 100ngr/ml ActivinA. Treatment of pluripotent cells by different concentrations of Rapamycin during the first day of induction can accelerate the differentiation trend of cells into DE. This treatment can cause remarkable decrease in expression of stemness genes like Oct4. Immunochemistry staining of sections of spheres confirmed the expression of SOX17 and FOXA2 proteins. This approach can inspire researchers to launch a new insight in practical cell-based therapies.

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Poster Board Number: 3144

MODELLING PROTEIN AGGREGATION IN MACHADO JOSEPH DISEASE USING PATIENT SPECIFIC IPS CELLS

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Machado-Joseph Disease (MJD; syn. spinocerebellar ataxia type 3; SCA3) is a dominantly inherited late-onset neurodegenerative disorder caused by expansion of polyglutamine (polyQ)-encoding CAG repeats in the MJD1 gene. Proteolytic cleavage of the MJD1 gene product, ataxin-3 (ATXN3), is believed to trigger the formation of ATXN3-containing aggregates, the neuropathological hallmark of MJD. Here we report the generation of induced pluripotent stem (iPS) cell-derived neurons from a patient with MJD and his non-affected sibling. We demonstrate that L-glutamate-induced excitation of MJD neurons is sufficient to initiate cleavage of ATXN3 and the formation of SDS-insoluble aggregates. Aggregate formation was efficiently blocked by the calpain inhibitors N-acetyl-Leu-Leu-norleucinal (ALLN) and calpeptin but not by caspase inhibition, indicating that excitation-induced activation of calpains is sufficient to generate aggregation-competent proteolytic polyQ fragments. Thus, our study illustrates the powerful potential of iPSC-derived cellular models to identify fundamental pathophysiological processes in human neurodegenerative disorders.

Poster Board Number: 3146

REDUCED LEVELS OF VAPB IN MOTOR NEURONS FROM ALS8 PATIENTS FOLLOWING IPSC FIBROBLASTS REPROGRAMMING

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ALS is a devastating and incurable neuromuscular disease that leads to a profound loss of life quality. Around 10% of the ALS cases are inherited. ALS8 is an autosomal dominant form of familial ALS, which is caused by mutations in the VAPB gene. It was first described in Brazilian patients and identified in other countries such as Germany, Japan and UK. The VAPB protein affects many cellular processes and likely contributes to the pathogenesis in many other forms of ALS besides ALS8. A substantial number of successful drug tests in ALS animal models could not be translated to humans demonstrating the need to develop other ALS model systems. Induced pluripotent stem-cells (iPSC) technology brings new hope in this area since it can be used to model and investigate diseases *in vitro*. Here we present a new ALS model based on ALS8-iPSC. Fibroblasts from 7 individuals, 4 patients and 3 non affected siblings from two ALS8 families, were successfully reprogrammed to a pluripotent state, accessed by the expression of embryonic stem cell markers. When injected into nude mice, these cells generated teratomas containing tissues from the three embryonic germ layers, showing that these arebona fide iPSC. We show, for the first time, that the VAPB protein is present in iPSC and in two different human embryonic stem cell lineages. Additionally, this is the first description that VAPB is reduced in fibroblasts, iPSC and differentiated motor neurons derived from ALS8 samples when compared to controls obtained from the normal siblings. We suggest that with aging the levels of VAPB may be crucial for motor neuron viability, supporting previous studies showing that VAPB is diminished in postmortem sporadic ALS samples. Besides helping in the understanding of motor neuron diseases, iPSC modeling will probably become a useful tool to answer why proteins expressed early in development cause late onset diseases.

Poster Board Number: 3148

INDUCED PLURIPOTENT STEM CELLS (IPS CELLS) - DERIVED HEPATOCYTES TRANSPLANTATION AS A THERAPEUTIC STRATEGY FOR GENETIC LIVER DISEASE

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Background: Recently, hepatocyte transplantation has been demonstrated as an effective therapeutic strategy for different kinds of liver diseases in animals and clinical trials, including metabolic liver disease, acute liver failure, and chronic liver cirrhosis. But the shortage of donor cells is still a big problem to overcome before the wide application of cell transplantation. The iPS cells have the potential to expand the donor cell source of hepatocyte transplantation for liver diseases. We will identify in this study whether the iPS cells - derived hepatocytes transplantation can be an effective therapeutic strategy for genetic liver diseases - hemophilia B as an example. Methods: In this study we plan to identify if the iPS cells can be a new source of donor cells. The iPS cells were provided kindly by Prof. YamanaKa S. Firstly, we will identify the capacity of iPS cells to differentiate into functional hepatyocytes. We applied the modified three steps protocol to differentiate the iPS cells into hepatocytes. The morphology and functional characteristics of iPS cells -- derived hepatocytes will analyze by microscope, RTPCR for hepatocytes-related genes (α-FP, factorIX, CK18, G6P, TAT, alb, HNF4, cyp2b, cyp7a, etc) expression, ELISA for hepatocytes-related protein secretion (albumin), cytochrome P 450 enzyme activity and ICG uptake test. The fate of iPS cells- derived hepatocytes also evaluated at different time points after differentiation (3, 7, 10, 14, 21, and 28 days). Secondly, we studied the *in vivo* therapeutic efficiency of these iPS cells- derived hepatocytes. We transplanted intrasplenically the iPS cells- derived hepatocytes (1million cells/mice) into FIX knock-out mice. The blood samples were taken at day 5, 10, 15, and 20 to measure the clotting activity and ELISA for factor IX. The engraftments of transplanted hepatocytes were evaluated by labeling the transplanted cells with Quantum dots before cell transplantation and quantitative PCR. The hemostatic functional studies were evaluated by the thromboelastography (TEG). We also performed 2nd transplantation 7 days after the 1st transplantation to determine whether this approach increased the therapeutic efficiency. Results: The iPS cells - derived hepatocytes showed typical morphology of primary isolated hepatocytes under microscope. These iPS cells-derived hepatocytes also expressed the hepatocytes--related gene. We can detect the increased albumin secretion up to 6ng/ml after differentiation. The ICG uptake test, the cytochrome P450 enzyme activity test, and the PAS staining for glycogen storage disclosed in advance the functional characteristics of these iPS cells-derived hepatocytes. The recipients (n = 8-10) transplanted with these iPS cells-derived hepatocytes exhibited 1.75%, 1.59%, 1.10%, and 1.46% of FIX clotting activity at day 5, 10, 15, and 20, which is compatible with those (n = 2-4) transplanted with primary isolated hepatocytes from wild type mice (1.63%, 1.20%, 0.93%, and 1.22% of FIX clotting activity respectively). Conclusion: These studies demonstrated that hepatocytes transplantation is an effective therapeutic strategy for genetic liver diseases. And the iPS cells can be an alternative source for hepatocytes transplantation to overcome the problem of organ

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shortage. These results will help us to develop the therapeutic strategy of cells transplantation for genetic liver diseases.

Poster Board Number: 3150

PATIENT-DERIVED IPS CELLS WITH A H137L MUTATION IN RP9 GENE MODEL RETINITIS PIGMENTOSA

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Retinitis pigmentosa (RP) is the most common inherited human eye disease resulting in night blindness and visual defects. RP encompasses a number of genetic subtypes, which overlap in both etiology and phenotype. Retinitis pigmentosa-9 is caused by an autosomal dominant mutation in RP9 (PAP-1) gene. The mechanism whereby the mutation in ubiquitously expressed RP9 gene merely leads to retinal degeneration remains unknown. We obtained from two familiar patients with a H137L mutation in the RP9 gene and generated induced pluripotent stem (iPS) cells. We differentiated them into rod photoreceptor cells by using a sophisticated protocol and found that differentiated rod photoreceptor cells showed immunocytochemical features and electrophysiological properties. Cell number of the patient-derived rod cells with distinct mutation underwent loss *in vitro*. We showed the cells derived from patients expressed markers of oxidative stress and were rescued by vitamin E. In conclusion, RP9 patient-derived rod cells recapitulated the disease phenotype. Using patient-derived iPS cell technology, our results provide insights for elucidation of pathogenic mechanisms caused by RP9 H137L mutation.

Poster Board Number: 3152

HUMAN EYE STROMAL CELLS AS NEW RESOURCES TO GENERATE HUMAN INDUCED PLURIPOTENT STEM CELLS (HIPSCS)

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The iPS cells have been seriously attended in last five years for therapeutic implications because of potentially surmounting of two important problems related to hES cells: 1) potency of immune rejection after transplantation, 2) ethical issues in terms of embryo destruction. Many investigations showed efficient establishment of iPS from different cells such as Fibroblast, stromal- and adult stem cells originated from different tissues. Here, we evaluate the possibility and efficiency of iPS derivation from human eye stromal cells. To end that, stromal segments of conjunctiva and trabecular meshwork biopsies were cultured and fibroblast-like stromal cells, which can be differentiated toward the osteogenic, adipogenic, chondrogenic, and neurogenic lineages, were isolated. Then the Eye-derived stromal cells were directly reprogrammed by either constitutive or doxycycline inducible lentiviral systems overexpressing four (Oct4, Sox2, Klf4 and c-Myc) or three (Oct4, Sox2, Klf4) key transcription factors. After three weeks, ES-like colonies were picked up and subcultured on MEF cells and were analyzed by PCR for stemness markers, immunocytochemistry for ES markers such as Nanog, Tra-1-60 and SSEA4, EB formation and Karyotyping. The pluripotency of ES-Like colonies was confirmed *in vitro* and *in vivo* by differentiation to all three germ layers lineages and teratoma formation in nude mice, respectively. In conclusion, we demonstrated that human eye-stromal cells, which can be obtained in ophthalmic surgery, can directly be reprogrammed to induced pluripotent stem cells like fibroblasts by either constitutive or inducible systems and interestingly the efficiency was the same (0.01%) as fibroblasts. As iPS derived from human eye-stromal cells may have maintained their genome imprinting, the cells may efficiently re-program to different cell types of eye tissue and provide unique resources for eye tissue regeneration.

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COMBINED INFLUENCES OF MATRIGEL AND ELECTROSPUN PLGA NANOFIBROUS SCAFFOLDS ON DIFFERENTIATION OF PLURIPOTENT MOUSE EMBRYONIC STEM CELLS

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In this study, we analyzed the effect of a natural Extra Celluar Matrix, Matrigel, in combination with synthetic/biodegradable electrospun PLGA-nanofibrous scaffolds on differentiation program of the stem cells. To end that, a GFP-expressing mouse embryonic stem (GFP-ES) cell line were generated by lentiviral gene transduction system and was used to study of all possible cell programming fats as well as monitoring of the attachment, growth and behavior of the cells on the scaffolds. The GFP-ES cells were characterized by RT-PCR, and immunocytochemistry for pluripotency markers such as Oct4 and Nanog. The PLGA nanofibrous scaffolds were fabricated by different parameters and characterized by SEM, FTIR-ATR and Atomic Force Microscopy (AFM). Then, ES- differentiating cells were seeded on PLGA scaffolds and the Matrigel was added as On-top embedding. The transcriptome of the differentiated cells was analyzed for expression of early developmental markers for endoderm, mesoderm and ectoderm and their late derivates such as specific marker for neuron, cardiomyocyte, hepatocyte, endothelia, adipocyte, muscle etc. as well as germ and ES cell markers using quantitative RT-PCR for more than fifty tissue-specific transcription factor/marker. The attachment, infiltration and morphological changes were monitored by SEM and IF microscopy. In conclusion we found that combination of Matrigel with PLGA scaffolds decrease the endodermal cell lineages programming whereas it promotes significantly mesodermal and germ cell lineages programming. The pluripotency status of the cells was maintained more in Matrigel/ PLGA combination condition compared to the cells cultured on scaffolds alone. The ectodermal programming of ES cells was more influenced by the roughness and alignments of scaffolds mats than combination with Matrigel.

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REPROGRAMMING OF MOUSE AND HUMAN SOMATIC CELLS BY HIGH PERFORMANCE ENGINEERED FACTORS

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Reprogramming somatic cells to induced pluripotent stem (iPS) cells by defined factors represents a major breakthrough in biology and medicine, yet remains inefficient and poorly understood at this stage. We therefore devised synthetic factors by fusing the VP16 transactivation domain to Oct4 (also known as Pou5f1), Nanog and Sox2 respectively. These synthetic factors could reprogram both mouse and human fibroblasts with enhanced efficiency and accelerated kinetics. Remarkably, Oct4-VP16 alone could reprogram mouse embryonic fibroblasts (MEFs) efficiently into germline-competent iPS cells. Furthermore, episomally- delivered synthetic factors could generate integration-free iPS cells from MEFs reproducibly with enhanced efficiency. Our results not only demonstrate the feasibility of engineering more potent reprogramming factors, but also suggest that transcriptional reactivation of Oct4 target genes might be a rate-limiting step in the conversion of somatic cells to pluripotent ones. Synthetic factor-based reprogramming may lead to a paradigm shift in reprogramming research.

Poster Board Number: 3158

EFFICIENT DERIVATION OF MOUSE INDUCED PLURIPOTENT STEM CELLS IN STIRRED SUSPENSION BIOREACTORS

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The generation of induced pluripotent stem cells (iPSCs) is an inefficient process, which requires several weeks before a cell line is established. Here, we report that iPSCs can be generated more quickly and efficiently in stirred suspension bioreactors (SSBs). We have previously shown that SSBs provide a homogenous and dynamic environment, suitable for expanding mouse and human embryonic stem cells (ESCs). Our work suggests that SSBs favour the maintenance of pluripotency over differentiation. In this study, we examined whether SSB culture presents a selective advantage in iPSC derivation. We transduced mouse embryonic fibroblasts (MEFs) with retroviral vectors of the four reprogramming genes (Oct4, Sox2, klf4 and c-Myc). Two days after transferring to a 100mL SSB at 100RPM, the MEFs formed aggregates, which were morphologically similar to ESC aggregates. These bioreactor-derived iPSC (BiPSC) aggregates expressed alkaline phosphatase (ALP) only 5 days post-transfection, whereas ALP expression was totally absent in the control static culture condition. We observed the *de novo* expression of major pluripotency markers such as Oct4, Nanog, SSEA1, Rex1, Dax1 and E-Ras on day 10. We were able to expand the culture to more than 50 million cells in less than 10 days, which is an outstanding difference, compared to conventional static culture methods.We observed about 2.5×105 ALP+ aggregates (i.e. iPSC colony equivalents) from an input of 5×105 original fibroblasts, suggesting an efficiency of 50%. This number is substantiated by our observation that approximately 50% of the cells in the bioreactor were ALP+ on day 12. BiPSCs from day 12 were karyotypically normal and could spontaneously differentiate into all three germ layers both *in vitro* and *in vivo*. We have recently generated several high percentage chimeras using BiPSCs and are awaiting the results of germline transmission. Our results suggest that liquid shear stress plays an important mechanistic role in bioreactor induced pluripotency. When we examined the nuclear translocation of β-catenin in ESCs, using the TCF/LEF GFP reporter system, we observed that considerably more β-catenin resides in the nucleus of cells undergoing liquid shear stress. We suggest that suspension cultures provide a selective advantage in enhancing iPSC generation partly by inducing nuclear β-catenin activation. Our results show for the first time that fibroblasts can be efficiently reprogrammed in SSBs. Combined with new methods of reprogramming that only use epigenetic reprogramming factors, our BiPSC technology has the potential to accelerate and standardize iPSC research, bringing it to clinical application more quickly.

Poster Board Number: 3160

ECTOPIC REPROGRAMMING FACTOR EXPRESSION AFFECTS THE EPIGENETIC STATUS OF THE IMPRINTED DLK1-DIO3 GENE CLUSTER IN MOUSE IPSCS

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Recent studies indicate that the epigenetic status of a single imprinted region, the Dlk1-Dio3 gene cluster, is sufficient to predict the developmental potential of induced pluripotent stem cells (iPSCs). For example, iPSC clones exhibiting epigenetic silencing of Gtl2, a member of the Dlk1-Dio3 cluster normally expressed from the maternally inherited allele, contribute poorly to chimeras and fail to produce viable mice through tetraploid complementation. To date, it has not been clear whether aberrant Gtl2 silencing in iPSCs results from the selection of a subset of previously mis-imprinted parental fibroblasts or occurs at some point during the reprogramming process. We have mapped the ontogeny of the epigenetic state of this locus, exemplified by the methylation state of CpG islands in the Gtl2 'intergenic differentially methylated region' (IG-DMR) in parental fibroblasts and in their iPSC progeny before vs after withdrawal of exogenous reprogramming factors (OKSM). Similar to tail tip fibroblasts (TTF) that exhibit ~50% methylation of the IG-DMR, in iPSCs reprogrammed with a single lentiviral copy of the STEMCCA-loxP vector, which combines all 4 factors in a single polycistronic transcript, we found 10 out of 10 TTF-derived iPSC clones (passage ~12-16) to be normally methylated at this locus (40-50%) in the presence of continued over-expression of OKSM. Following Cre-mediated excision of STEMCCA-loxP, resulting in withdrawal of exogenous OKSM, the majority of iPSC clones maintained a normal ESC-like methylated IG-DMR state, and 2 out 10 became aberrantly imprinted (hypermethylated). To further evaluate the global molecular changes associated with withdrawal of exogenous OKSM, gene expression profiling was performed on these 10 iPSC clones both before and after STEMCCA-loxP vector excision. Hierarchical clustering and PCA analyses indicated that after removal of exogenous factors, the global gene expression program of all 10 iPSC clones clustered more closely to control ESCs. Remarkably, no significant differences (FDR-adjusted $p<0.01$) were found between iPSCs reprogrammed with three factors (OKS; 5 clones) vs. four factors (OKSM; 5 clones). Our findings provide insights into the molecular events that occur during reprogramming and suggest that normal vs aberrant imprinting of the Dlk1-Dio3 domain in iPSCs occurs following the withdrawal of exogenous reprogramming factors.

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IMPLICATION OF INFLAMMATORY SIGNALING IN SOMATIC CELL REPROGRAMMING IN MOUSE

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Achievement of pluripotency through somatic cell reprogramming with defined factors provides not only source of regenerative medicine but also tool to investigate the developmental cues for ontogeny of various cell types. Despite success of induced pluripotent stem cells (iPSCs) establishment, the molecular processes associated with somatic cell reprogramming are still poorly understood. Our laboratory has established iPSCs by combining piggyBac transposons for delivery of the reprogramming transgenes with the doxycycline inducible system. A unique feature of these iPSC lines is that their differentiated derivatives (2° MEFs) can be sent back to a pluripotent state in a population manner through reactivation of the reprogramming transgenes by administration of doxycycline. Transcriptome profiling during the three-week course of the reprogramming process revealed 1) mesenchymal to epithelial transition (MET) reflected by gradual down regulation of mesenchymal and up regulation of epithelial markers, and 2) a temporal increase in the expression of interferon (IFN) response genes. Here we present evidence that IFN related inflammatory signaling is implicated in somatic cell reprogramming, and its pharmacological activation or inhibition result in enhanced or reduced colony formation, respectively. This study provides comprehensive data on the potential involvement of the inflammation-response pathway driving reprogramming of somatic cells, broadening our understanding on molecular basis of pluripotency and development, and facilitating safe and personalized regenerative medicine.

Poster Board Number: 3164

GENERATION OF STABLE PLURIPOTENT STEM CELLS FROM ADULT NON-OBESE DIABETIC MICE

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The nonobese diabetic (NOD) mouse strain has been widely used to investigate the pathology and genetic susceptibility for type 1-diabetes. Induced pluripotent stem cells (iPSCs) derived from this unique mouse strain would enable novel strategies for investigating T1D pathogenesis and potential therapeutic targets. The objective of this study was to determine if somatic fibroblasts from NOD mice could be reprogrammed to iPSCs. Adult tail-tip fibroblasts from male NOD mice were reprogrammed by retroviral transduction of the coding sequences of three transcription factors, OCT4, SOX2 and KLF4, in combination with a histone deacetylase inhibitor, valproic acid (VPA). Three of the 18 NOD iPSC lines generated were characterized and exhibited silencing of the reprogramming transgenes and reactivation of endogenous pluripotent markers (OCT4, SOX2, NANOG, REX1 and SSEA1). The iPSCs readily differentiated *in vitro* to form embryoid bodies, and *in vivo* by teratoma formation in immuno-deficient mice. Moreover, the cells were successfully transfected with a reporter transgene, and were capable of contributing to the inner cell mass of recipient blastocysts, leading to the generation of a chimeric mouse. In conclusion adult tail-tip fibroblasts from NOD mice can be reprogrammed, without constitutive ectopic expression of transcription factors, to produce pluripotent iPSCs, which can be maintained and propagated under normal ESC culture conditions to produce genetically altered cell lines, differentiated cells and chimeric mice.

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GENERATION OF MOUSE INDUCED PLURIPOTENT STEM CELLS FROM DIFFERENT GENETIC BACKGROUNDS BY EXCISABLE LENTIVIRAL SYSTEM

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The long-term goal of induced pluripotent stem (iPS) cell technology is to generate patient specific donor cells for transplantation, which can be expanded and differentiated to multiple directions, and also genetically modified for gene therapy purposes. The scientific aim of this study is to further develop techniques for generation therapeutically safe iPS cells. For generation of iPS cells we used a novel polycistronic lentiviral gene delivery system to minimize the risk of insertional mutagenesis caused by multiple insertion sites. We also inserted a LoxP site in our expression construct to make the integrated transgenes removable from the genome. To test if our construct is able to reprogram embryonic fibroblast cells from different genetic backgrounds we tested it on two inbred (C57BL/6 and C57BL/6xDBA/2J) and one outbred (CD1) mouse strain. The lentiviral delivery was optimized for low copy integration. The iPS cell lines were characterized by morphology, RT-PCR and immunostaining (IHC) for pluripotency markers, such as alkaline phosphatase, Pou5f1/Oct4, Nanog and SSEA-1. We differentiated the cells *in vitro* into cardiac and neural lineages. The number of viral integrations was determined by quantitative PCR. The reprogramming efficiency was approximately 0.01%. We found that the morphology of our iPS lines were very similar to normal embryonic stem (ES) cells and also the iPS cells expressed all examined pluripotency markers. The iPS clones after Cre-excision were able to differentiate into beating cardiomyocytes (70±10%) *in vitro*. Also, the reprogramming factor free clones differentiated to neural lineage, which was confirmed by IHC for neural progenitor cells (nestin) and for mature neural markers (βIII-tubulin). In both *in vitro* differentiation assays we found that the iPS clones before Cre-excision have weak differentiation ability, while after removing the reprogramming factors they were able to differentiate comparable to standard ES cells. The *in vivo* chimera-formation experiments from the reprogramming factor free cell lines are in progress. In conclusion, we have successfully derived iPS cells from different genetic backgrounds. After deletion of all reprogramming factors, the iPS lines maintained their pluripotency and showed characteristics more similar to normal ES cells than the iPS lines containing the transgenes. The generation of iPS cells with an excisable lentiviral construct represents a promising technology and their *in vitro* differentiation to different cell types provide a good opportunity for studies on cell replacement therapy and pharmacological testing.

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C-MYC PLAYS A CRUCIAL ROLE IN GENERATING FULL DEVELOPMENTAL POTENTIAL OF IPSCS

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iPSC was initially generated by the introduction of four transcription-regulation factors, OSKC, into mouse embryonic fibroblasts with retrovirus gene transduction system, and dispensability of c-Myc for the generation was subsequently shown. Therefore, it has been believed that there is no significant differences between iPSCs generated using the 4 factors and the 3 factors excluding c-Myc. Here we carefully investigated two types of iPSCs focusing on their differences. To exclude the effects of their genetic backgrounds and of the possible aberrations caused by the integration of vectors we used genome integration-free iPSCs generated from an inbred strain, C57BL/6J. As reported, few significant differences were observed when we conducted molecular biological analyses on transcripts, DNA methylation state, genes products. Test for the teratoma formation also showed no difference between the two types of iPSCs. Subsequently, we conducted an aggregation test for evaluating their developmental abilities. Experiments using three of 4FiPSC clones and five of 3FiPSC clones revealed that although 4FiPSCs developed to highly chimeric mice efficiently and showed frequent germline transmission, few 3FiPSCs could develop to highly chimeric mice, indicating that 4FiPSC clones have full developmental potential compared to 3FiPSC clones. Similar incompleteness was also observed in the 3FiPSCs generated by another procedure, with retrovirus gene transduction system, and by using another mouse, Nanog-TG mouse, that has been frequently used for iPSC studies thus far. Interestingly, even if highly chimeric mice were born from the embryos aggregated with 3FiPSCs, they died soon after their birth. In addition, we also found a difference in the implantation frequency of the aggregated blastocysts between the two types of iPSCs. Therefore, we directly investigated the aggregation steps using GFP-labeled iPSCs with time-lapse system and then revealed a defect in aggregation process of 3FiPSCs. The observation suggested that the molecular difference could be detected during *in vitro* blastocysts development. We therefore performed comprehensive analyses of these iPSCs for transcriptome and methylome. While no significant difference was shown in R-value between 4F and 3FiPSC clones, several transcripts and CpG sites that exhibited difference between them, were identified. Currently we are investigating these transcripts and genetic regions.

Poster Board Number: 3170

GENERATION OF MOUSE INDUCED PLURIPOTENT STEM CELLS BY PROTEIN TRANSDUCTION

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Somatic cell reprogramming have generated an enormous interest after the first report by Yamanaka and coworkers on the generation of induced pluripotent stem cells from mouse fibroblasts by expressing four defined transcription factors (Klf4, Oct4, Sox2 and c-Myc). To address the safety issues arose from harboring integrated exogenous sequences in the target cell genome, a number of methods have been developed and produced iPSCs with potentially reduced risks. Here we report the generation of stable induced pluripotent stem cells (iPSC) from mouse fibroblasts by treating them with purified proteins (Klf4, Oct4, Sox2 and c-Myc) carrying the GST tag for purification and the TAT-NLS polypeptide to facilitate membrane penetration and nuclear localization. We performed the reprogramming procedure on embryonic fibroblasts from two different genetic backgrounds: one inbred (C57BL/6xDBA/2J) and one outbred (ICR). The cells were treated four times with the purified proteins at an interval of 48 hrs between treatments. Following the treatment the cells were transferred onto mytomycin treated MEF and kept in complete medium until colonies formed. The reprogramming was successful just for the fibroblasts from the outbred strain. The newly generated iPSC have similar morphology to the mouse embryonic stem cells (ESCs) they can be sustained undifferentiated for over 20 passages. The cells were checked for the typical pluripotency markers (Nanog, Oct4, Sox2, Klf4, cMyc, APS) by immunocytochemistry. The RT-PCR analysis confirmed endogenous gene expression of a number of pluripotency genes. To verify the developmental potential of the protein IPS cells we performed standard *in vitro* differentiation by embryoid body (EB) formation, and *in vivo* chimerism assays. The pIPS cells formed EBs in suspension and subsequently differentiated towards cardiac and neural lineages. The pIPSC differentiated into cardiomyocytes started to beat on day 6 of differentiation. On day 7 almost all cardiomyocytes (more than 80%) were beating. These EBs were cardiac Troponin T and Troponin I (marker of contracting muscle) positive. The pIPS were also positive for Nestin (early neural marker) and Tuj1 (neuron specific class III beta Tubulin, mature neural marker). Approximately 40% of the cells seeded differentiated into neural precursor cells (NPC). Importantly the pIPS cells could incorporate into the blastocyst and led to high degree of chimerism in new born mice. These data show that the recombinant purified cell-penetrating proteins are capable to reprogram mouse embryonic fibroblasts to iPSC showing similar morphology and functional behavior as the bona fide ESC.

Poster Board Number: 3172

THE DERIVATION OF FUNCTIONAL NEURONS FROM MOUSE INDUCED PLURIPOTENT STEM CELLS IS ENHANCED BY EXTENDED PASSAGING

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Cellular reprogramming is a promising approach to deriving specific neuronal populations lost due to disease or injury-related neurodegeneration. Unfortunately, the full extent of genomic and epigenomic changes that occur during the reprogramming process are poorly understood. Thus far, the capacity of mouse and human iPSC lines to differentiate into neurons has been highly variable, prompting the need for a reliable means of assessing the differentiation capacity of newly derived cell lines. Extended passaging is emerging as a method of ensuring faithful reprogramming. We adapted an established and efficient embryonic stem cell (ESC) neuronal induction protocol to test whether iPSCs (1) have the competence to give rise to functional neurons with similar efficiency as ESCs and (2) whether the extent of neuronal differentiation could be altered or enhanced by extended time in culture prior to differentiation. Our initial gene expression and morphological analyses revealed that neuronal conversion was temporally delayed in iPSC lines and some iPSC lines did not properly form embryoid bodies during the first stage of differentiation. Notably, these deficits were corrected by continual

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passaging in an iPSC clone. Late-passage iPSCs expressed higher mRNA levels of pluripotency markers and formed larger embryoid bodies than the early-passage iPSCs. Moreover, late-passage iPSCs expressed neural marker genes several days in advance of early-passage iPSCs after the start of neural induction. Furthermore, late-passage iPSC-derived neurons exhibited notably greater excitability and more mature membrane properties than early-passage iPSC-derived neurons, although these cells were morphologically indistinguishable. These findings demonstrate that highly efficient neuronal conversion is critically dependent on the complete reprogramming of iPSCs via extensive passaging.

Poster Board Number: 3174

MOUSE DEFINITIVE NEURAL STEM CELLS GENERATION FROM PIGGYBAC TRANSPOSON INDUCED PLURIPOTENT STEM CELLS CAN BE ENHANCED BY INDUCTION OF THE NOTCH SIGNALING PATHWAY.

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INTRODUCTION: Despite advances in medical and surgical care, current clinical therapies for spinal cord injury (SCI) have limited effectiveness. Cell-based therapies using neural stem cells (NSC) animal models of SCI have shown positive outcomes. Although, efforts are currently underway to translate the use of NSC derived from embryonic stem (ES) cells for SCI, the clinical application of these technologies have been limited by a number of potential concerns. The discovery of induced pluripotent stem (iPS) cells holds the promise to revolutionize the field of regenerative medicine by addressing many of these caveats, including the host immune response to allogeneic cells and availability. However, in light of recent developments on the inherent differences between ES and IPS cells, particular in terms of epigenetics, detailed characterization of the iPS-derived NSCs will be require before translation experiments can be performed. METHODS: Murine embryonic fibroblast are transfected with a piggyBac transposon plasmid contain all of the reprogramming factors (Oct4, Sox2, Klf4, c-myc). Following induction of pluripotency the reprogramming factors are seamlessly excised. The PB-iPS cells were clonally expanded along the default pathway of neutralization to generating NSCs initially with serum free media (SFM) containing LIF (primitive NSC; pNSC) and followed by SFM with FGF (definitive NSC; dNSC). ES cells (R1) were used a control cell line for comparison. RESULTS: Primitive and definitive neursosphere were successfully generated using the default pathway condition with the iPS cell qualitatively similar to the ES cell. The iPS-derived dNSCs showed increases in neural specific marker (Pax6, Nestin, Olig2, Map2). However, the iPS-dNSC express significantly higher levels of pluripotency (Oct4, Nanog, lin28) and non-ectoderm lineages (Gata4, Gata6, Afp) genes compared to equivalent ES-dNSCs. Furthermore, *in vitro* differentiation of the ES-dNSC show a restricted neural fate, while only a subset of the differentiated iPS-dNSC stained for neural cell markers. The neuralization of the PB-iPS cells can be improved by reinforcing the default conditions. The addition of Noggin (a BMP antagonist) to the culture media significantly increase primary neurosphere generation in the iPS cell lines. Also, the induction of the NOTCH signaling pathway by Delta-like ligand 4 (DLL4) improved the transition from primitive (LIF-dependant) NSCs the definitive (FGF-dependant) state. CONCLUSION: This study suggests that intrinsic differences between ES and iPS cell can affect their ability to acquire a dNSC fate. However by manipulating critical stages of the neuralization with recombinant proteins, the IPS cells can be forced to a restricted neural identity. Given the increasing recognition of key differences between ES and iPS cells, the development of methods that can overcome these differences will be required to realize the full potential of iPS cells.

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DIFFERENTIATED RETT SYNDROME MOUSE INDUCED PLURIPOTENT STEM CELLS REVEAL NOVEL IMMATURE NEURON PHENOTYPES

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Induced pluripotent stem (iPS) cells hold great promise for studying central nervous system disorders *in vitro*, whereby neuronal differentiation of patient-specific iPS cells creates a limitless supply of defective neurons for disease study. Rett Syndrome (RTT) is a neurodevelopmental autism spectrum disorder primarily caused by mutations in the methyl CpG-binding protein 2 (MECP2) gene. Due to the inaccessibility of patient neurons, most of what is known about underlying RTT phenotypes has been described using mouse models. Here we describe the characterization and neuronal differentiation of mouse RTT iPS cell lines. Wild-type and heterozygous iPS cell lines express endogenous pluripotency markers, reactivate the X-chromosome, and differentiate into the three germ layers. Directed differentiation of the lines produced neurons which formed functional synapses that generate action potentials and miniature excitatory postsynaptic currents (mEPSCs). Consistent with previous reports in MeCP2-null mouse brain slices, neurons derived from MeCP2-deficient iPS cells reproduce defects in the generation of evoked action potentials and mEPSC frequency. Further, they exhibit novel phenotypes when compared to wild-type iPS-derived neurons, specifically, diminished peak inward and outward currents, higher average input resistances, and more depolarized resting membrane potentials. Taken together, these results indicate that MeCP2-deficient iPS-derived neurons recapitulate previously described defects observed in MeCP2-null mouse neurons and exhibit novel immature neuron electrophysiology with minimal variability between lines. These newly identified phenotypes are consistent with the hypothesized role of MeCP2 in modulating neuronal maturity, and provide additional maturation deficiencies that reveal novel insight into RTT pathogenesis.

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NT AND PARTHENOGENESIS EMBRYO CHIMERA BECOMES A CRITERION FOR IDENTIFYING THE NATURE OF PIG INDUCED PLURIPOTENT STEM CELLS

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Without pig embryonic stem (ES) cells highly restricted the usage of pig as an ideal model in medical research and the production of transgenic animals. Reprogramming of somatic cells into iPS cells *in vitro* had been achieved through over expression of defined transcription factors in 2006. Then, pig iPS cells were established in 2009. However, pig iPS cells generated from the different labs have showed the different characteristics, and were not yet obtain the germline transmission chimera. In this study, we generated pig iPS cells by the retrovirus system using pMXs vectors containing mouse 4F (Oct4\Sox2\KLF4\c-Myc). Four established cell lines can grow stably on MEF feeder in Knock-out DMEM with 15% FBS plus both LIF and bFGF at 38.5°C, 5% CO2 atmosphere, and have been continually cultured for over 30 passages without changing the karyotype. The morphology of isolated piPS cells is more like mouse ES cells rather than human ES cells. The piPSs express Oct4, Sox2, Nanog, TERT and ES cells specific markers (SSEA1, SSEA4, Tra-1-60 and Tra-1-81) and show the activity of alkaline phos-

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phatase (AP). Meanwhile, the ES cell specific signal receptors LIFR, bFGFR and SCFR were s also significantly reactivated. However, we found that the expression of 4 exogenous factors in these piPS cell lines were not silenced, similar to the results reported in the other labs. To evaluate the pluripotency of piPS cells, the nuclear transfer (NT) embryo and parthenogenesis embryo chimera were applied for the determination of developmental potential of pips cell lines. The result showed that the high expression of 4 exogenous factors could seriously block NT embryo development, which was mostly stopped in 4-8 cell stage, and barely to develop into blastocytes. We have injected 1-2 piPS cells (labeled with red fluorescent protein) into parthenogenesis embryo to detect the chimeric potential of piPS. The results showed that piPS cell line with the normal karyotype and lower expression of exogenous factors could create the chimeric embryo.

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EVALUATION OF REPROGRAMMING EVENTS IN A SINGLE PORCINE INDUCED PLURIPOTENT STEM CELL-LIKE LINE.

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Previous research in production of porcine induced pluripotent stem cells (piPSC) reveals that transgenes are unable to be appropriately silenced in this species. Despite this revelation, differentiation of piPSC can be achieved. This lack of transgene silencing is an interesting phenomena and should be further investigated. In this study, we closely analyzed both exogenous and endogenous gene expression of OCT4, NANOG, SOX2, KLF4 and cMYC in a putative porcine induced pluripotent stem cell line during extended in-vitro culture. An inducible expression system of piPSCs was produced by lentivirus transduction using the human reprogramming factors (OSMN*K) (*NANOG pseudogene P8) as obtained from Addgene. A single colony was isolated 7 days following transduction and sub-cultured in KO-DMEM + 15% KSR, 1 x NEAA, 1x pen/strep 0.1mM beta-mercaptoethanol, 20ng/ml human recombinant bFGF and either 1ug/ml doxycycline or 4ug/ml doxycycline. Cells were maintained on mitomycin c-treated mouse embryonic fibroblasts and mechanically passaged every 4-5 days. Colonies were isolated at Passage 5, 10, 15 and 20 and RNA was isolated using the Qiagen RNeasy mini kit and cDNA amplified using the Quantitech whole transcriptome kit respective. Semi-quantitative RT-PCR was performed to analyze endogenous and exogenous gene expression of OCT4, NANOG, SOX2, KLF4 and cMYC using customized primer sets. Protein expression of OCT4, NANOG, SOX2, KLF4 and cMYC was also analyzed by immunocytochemistry, in early passage cells. Results indicated that all transgenes could be detected with the exception of SOX2. Interestingly, protein expression revealed SOX2 was present at Passage 1 and 3. Endogenous SOX2 could also be detected in cells grown using 1ug/ml doxycycline, but not 4ug/ml doxycycline. Genomic PCR confirmed the lack of the human SOX2 transgene. The endogenous SOX2 expression was inhibited when cultured in 4ug/ml doxycycline. In addition, endogenous gene expression did occur for NANOG KLF4 and cMYC, but was delayed until passage 10 in the high doxycycline cell cultures. Evaluation of OCT4 endogenous expression was ambigious. We observed a single band of incorrect size in our piPSC-like cells, which when sequenced did not correspond to OCT4. This was not observed in our control tissues (porcine epiblast). This primer set is designed to amplify the stem-cell specific isoform OCT4A, and we wonder if unknown splicing may interfere in amplification of this gene. This piPSC-like line could not be maintained in an undifferentiated state in the absence of doxycycline. Further this cell line could readily form embryoid bodies in-vitro, but only in the presence of doxycycline. Better quality and larger embryoid bodies formed in 4ug/ml doxycycline. In conclusion, endogenous expression of porcine NANOG, SOX2, KLF4 and cMYC could not replace human transgenes and sustain proliferation in-vitro. Endogenous expression of porcine OCT4 was lacking and may be crucial for reprogramming. The level of doxycycline expression also appears to be important for in-vitro culture and reprogramming. Further molecular analyses are required in order to understand the reprogramming events occurring in piPSC lines. This work was financed by EU FP7 grants (PartnErS, PIAP-GA-2008-218205 and PluriSyS, 223485).

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EXPRESSION OF A NON-VIRAL POLY-PROMOTER VECTOR INDUCES PLURIPOTENCY IN QUIESCENT BOVINE CELLS UNDER CHEMICALLY DEFINED CONDITIONS

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Induced pluripotent stem cells (iPSCs), capable of giving rise to all cell types of an adult animal, are only available in mouse. Here, we report the first generation of bovine iPSC-like cells following transfection with a novel non-viral poly-promoter vector. This vector contains the bovine cDNAs for OCT4, SOX2, KLF4 and c-MYC, each controlled by its own constitutive promoter. Following transfection, bovine fibroblasts were cultured without feeders in a chemically-defined medium containing leukaemia inhibitory factor and inhibitors of Erk 1/2 signalling (PD0325901) and glycogen synthase kinase-3 (CHIR99021). Non-invasive real-time kinetic profiling revealed a different response of bovine vs human and mouse cells to culture in PD/CHIR-supplemented media. Only in bovine, PD/CHIR was necessary and sufficient to induce the appearance of tightly packed alkaline phosphatase-positive iPSC-like colonies. These colonies formed in the absence of DNA synthesis and did not expand in 2i/Lif after passaging. Non-proliferative primary colonies expressed discriminatory markers of ground state pluripotency, including endogenous iPS factors, NANOG, SALL4, DPPA3, telomerase activity, Tra-1-60/1-81 and SSEA-3/4, but not SSEA-1. This indicates that they had initiated a self-sustaining pluripotency programme. Bovine iPSC-like cells maintained a normal karyotype and differentiated into derivatives of all three germ layers *in vitro* and in teratomas. Our study demonstrates that conversion into induced pluripotency can occur in quiescent cells, following a previously undescribed route of direct cell reprogramming. This identifies a major species-specific barrier for generating iPSCs and provides a chemically-defined screening platform for factors that maintain proliferation and pluripotency of embryo-derived pluripotent stem cells in livestock.

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SAFEGUARDING NONHUMAN PRIMATE IPS CELLS WITH SUICIDE GENES

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The development of technology to generate induced pluripotent stem (iPS) cells constitutes one of the most exciting scientific breakthroughs because of the enormous potential for regenerative medicine. However, the safety of iPS cell-related products is a major concern for clinical translation. Insertional mutagenesis, possible oncogenic transformation of iPS cells or their derivatives, or the contamination of differentiated iPS cells with undifferentiated cells, resulting in the formation of teratomas, have remained considerable obstacles. Here, we demonstrate the utility of suicide genes to safeguard iPS cells and their derivatives. We found suicide genes can control the cell fate of iPS cells *in vitro* and *in vivo* without interfering with their pluripotency and self-renewal capacity. This study will be useful to evaluate the safety of iPS cell technology in a clinically highly relevant, large animal model and further benefit the clinical use of human iPS cells.

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PRE-CLINICAL EVALUATION OF PLURIPOTENT/ MULTIPOTENT STEM CELL BASED THERAPIES IN NON HUMAN PRIMATES.

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The transplantation of pluripotent and multipotent stem cells or their differentiated derivatives is a promising treatment strategy for degenerative diseases as well as other diseases requiring tissue replacement. To date, all experimental data on the safety and efficacy of cells derived from human ES/iPS cells *in vivo* derivatives is necessarily limited to xenotransplant models, specifically immunocompromised mice. The profoundly immunodeficient xenotransplant setting does not reflect clinical reality, and there are numerous physiological differences between humans and mice that limit the predictive value of this model. Unlike many rodent based models, the rhesus macaque (Macaca mulatta) is developmentally and physiologically closely related to humans and therefore represents a highly desirable model develop clinical applications for ES/iPSCs. Our study focuses on the production of bone from bone marrow mesenchymal stromal cells (BM-MSCs) and rhesus macaque induced pluripotent stem cells (rhiPSc) *in vivo*. Despite their clinical potential, the use of BM-MSCs is limited by their availability, restricted proliferation capacity and phenotypic heterogeneity. An alternative to BM-MSCs could be the use of autologous iPSc-derived MSCs. It has been shown that human BM-MSCs are able to form mature bone organs when injected along with a matrix consisting of hydroxyapatite/tricalcium phosphate (HA/TCP) particles in immunocompromised mice. In order to investigate if autologous and allogeneic rhesus BM-MSCs are also able to form bone structures in the rhesus macaque, we cultured marrow stromal cells derived from BM for 1-3 passages. Flow cytometric analysis demonstrated positive staining for CD105, CD166, CD73, CD90 and CD44, but as expected, the cells were negative for CD45 and CD34. Autologous and allogeneic BM-MSCs were incubated with 40 mg of HA/TCP particles for 90 minutes at 37C and transplanted into recipient animals subcutaneously. After 8-16 weeks, the organoids were harvested and histologically analyzed for signs of bone formation. Viable bone was observed within the autologous but not the allogeneic implants. To produce rhesus iPSCs, adult BM-MSCs were isolated, passaged four times and transduced with retroviral vectors carrying species-specific reprogramming transcription factors OCT4, SOX2, KLF4 and C-MYC. On Day 4, cells were plated onto mouse embryonic fibroblast feeder layers (mEFs) and cultured in ESC culture medium under 5% oxygen (KO-DMEM supplemented with 20% ES-defined FBS, 1% nonessential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and 20 ng/ml bFGF). ES-like colonies were picked approximately 21 days after transduction and expanded for further characterization. Generated rhiPSc lines morphologically resembled rhesus ES-cells (ORMES-22), stained positive for alkaline phosphatase, expressed endogenous pluripotency markers (Oct-4, Nanog, SSEA4, Tra1-60), and silenced viral reprogramming factors. Furthermore, rhiPSc spontaneously differentiated into derivatives of the three embryonic germ layers *in vitro*, and formed tumors in NSG mice. We differentiated the rhiPSc in the presence of bFGF, PDGF and EGF toward mesenchymal lineages. Similar to BM-MSCs, the derived cells stained positive for the surface markers CD105, CD166, CD73 and CD90 and negative for CD45 and CD34. Further *in vitro* characterizations as well as *in vivo* studies are ongoing and results will be discussed in the presentation.

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GENERATION OF GERMLINE COMPETENT RAT INDUCED PLURIPOTENT STEM CELLS

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Recent report showed that rat ES cells (rESCs) were established under the condition of using two or three kinds of kinase inhibitors including GSK3 inhibitor, MEK inhibitor and FGF receptor tyrosine kinase inhibitor in the culture medium and these rESCs possess an ability of generating chimeric rats and germline transmission. Soon after this report, establishment of rat induced pluripotent stem cells (riPSCs) that could contribute to chimera formation was reported. However, germline competency of riPSCs has not yet been reported. Here, we report for the first time, a successful generation of germline competent riPSCs and these riPSCs were able to generate interspecific germline chimera. Rat embryonic fibroblast cells (REFs) were infected with a lentiviral vector carrying mouse reprogramming factors (Oct4, Klf4 and Sox2) under tetracycline regulatory element and Ubc promoter driven EGFP as a marker. Infected REFs were seeded on mitomycin-C treated mouse embryonic fibroblasts (MEFs) and medium was changed to serum free medium (N2B27/F12) containing Dox and rLIF. MEK inhibitor (PD0325901) and GSK3 inhibitor (CHIR99025) (2i) or 2i and FGF receptor inhibitor (SU5409) (3i) were added to medium on day7. EGFP expressing mouse ES cell-like colonies appeared from day10 and independent colonies were picked. Picked riPSCs were expressing pluripotent marker genes at quantities comparable to those in rESCs and their ability to differentiate into three germ layers was confirmed by teratoma formation. To generate chimeric rats, we injected riPSCs into blastocysts from Wister (WI) rats and determined the formation of chimera by expression of EGFP in embryos or neonates. As a result, we obtained 11 chimeras (61 %) from riPSCs T1-3 (WI) and 7 chimeras (54 %) from riPSCs T3-11(WI) respectively. To address whether these riPSCs possess the ability of germiline transmission, generated male chimeraic rats were mated with wild type female WI/WI rats. We obtained three embryos (E15.5) and eight pups which express EGFP from three chimeric rats derived from riPSCs T1-3 or T3-11. Furthermore, we generated rat-mouse interspecific chimera and analyzed the expression of EGFP in embryonic fibroblasts. As a result, embryos derived from 16 out of 17 riPSCs clones had EGFP positive fibroblasts indicating that most riPSCs clones possess the ability to form interspecific chimera. Furthermore we analyzed to see whether riPSCs (T1-3) possess an ability to generate interspecific germline chimera by immunostaining of testis from mouse-rat interspecific chimera with antibody against mouse versa homolog (MVH) and the result showed that EGFP and MVH double positive germ cells were found in testis. These data provide conclusive evidence that rat embryonic fibroblasts can be reprogrammed into ground state pluripotent stem cells by three mouse reprogramming factors (Oct4, Klf4 and Sox2) and these riPSCs can differentiate into germ cells even in xenogenic mouse environment.

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GENERATION OF CANINE IPS CELLS AND THEIR CHARACTERISTICS

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For the clinical application of induced pluripotent stem cells (iPSCs) to regenerative medicine, there are many issues to be overcome. One of them is to establish appropriate non-rodent animal models for preclinical experiments, in which the efficacy and the safety of the iPSCs-based treatments can be monitored. For this purpose, dog is a suitable animal because of its long life and anatomical and physiological similarities with human. In addition, dog and human share some pathological conditions with identical genetic cause such as muscular dystrophy. We are currently working on the generation of canine iPSCs from somatic cells to take those advantages. As the cell source for generating iPSCs, embryonic fibroblasts were used. We have cloned canine Yamanaka's factors and other reprogramming factors, and constructed retroviral vectors expressing each factor. These factors were introduced canine embryonic fibroblasts, which demonstrated iPSC-like colonies after two to three weeks. We have attempted a series of culture conditions, and finally established an appropriate one for maintaining iPSC-like morphology of clones. Gene expression profiles of each clone showed clonal differences, but were basically similar to those of human and mice pluripotent stem cells. Characterization of each clone in terms of its differentiation property *in vivo* and *in vitro* is currently ongoing.

Poster Board Number: 3192

INVESTIGATION OF OPTIMIZED CONDITIONS FOR RNA BASED CELLULAR REPROGRAMMING

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Induced pluripotent stem (iPS) cells have been generated from somatic cells by forced ectopic expression of a set of pluripotency-associated transcription factors. They can grow indefinitely while maintaining pluripotency and differentiate into all three germ layers except for extra-embryonic tissues. This technology opened new ways to use individual-specific stem cells for regenerative medicine, avoiding ethical issues and immune rejections that human embryonic stem (ES) cells face. In terms of clinical application, generation of iPS cells using non-integrating methods which avoid the risk of insertional mutagenesis or genome modification are essential for therapeutic purposes. RNA-based cellular reprogramming is one of the most promising strategies to generate safe iPS cells, but cytosolic delivery of non-self RNAs coding for reprogramming factors induces innate antiviral responses with the rapid production of a range of cytotoxic cytokines such as interferons, resulting apoptosis of transfected cells. Here we investigated methodologies to optimize protocols to generate iPS cells with RNA-based cellular reprogramming, minimizing activation of cytoplasmic RNA sensors. We started with human adipose-derived stromal cells (ADS) cells and transfected *in vitro* transcribed RNAs coding for Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc) into ADS cells using nucleofection. In order to minimize activation of cytoplasmic RNA sensors and maximize translation efficiency, reprogramming RNAs were synthesized with modified nucleotides, capped with Anti-Reverse Cap Analog (ARCA) at the 5′ end, and polyadenylated at the 3′ end. Silencing of cytoplasmic RNA sensors with small interfering RNAs (siRNAs) was employed as adjuvants to suppress residual antiviral activity. We shall report progress of our research, mainly focusing on assay results. Our current progress will provide insight on the development of RNA-based cellular reprogramming and facilitate to establish safe iPS cells for clinical applications in regenerative medicine.

Poster Board Number: 3194

LIN28 ACCELERATES THE GROWTH OF REPROGRAMMING CELLS DURING IPS CELL INDUCTION

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Human induced pluripotent stem cells (iPSC) were established with OCT3/4, SOX2, KLF4, C-MYC (OSKM) or OCT3/4, SOX2, NANOG, LIN28 (OSNL). We found that OSKML generated 2~10 fold more iPS cell colonies than OSKM. LIN28 has 2 RNA binding domains, cold shock protein domain (CSD) and zinc finger protein domain (Zinc). Several reports indicated LIN28 regulated the stability of micro RNA through these domains. We made CSD or ZINC deletion mutants LIN28 and induced iPSC by introducing OSKM with those mutants. CSD deletion mutant increased the efficiency of iPSC induction. However, Zinc deletion mutant did not. These data suggested that LIN28 regulated the micro RNA through ZINC to increase the efficiency of iPSC induction. Moreover we focused on another phenomenon. The size of iPSC colonies made with OSKML was bigger than OSKM. There were 2 possibilities. One of them was that LIN28 brought forward the timing of generating iPSC. Another possibility was that LIN28 accelerated the growth of reprogramming cells. We carried out time laps imaging during iPS induction to test these possibility. The timing of arising iPSC colonies was not different between OSKML and OSKM. However growth of iPSC colonies with OSKML was faster than with OSKM. These data indicated that LIN28 accelerated the growth of reprogramming cells to increase the efficiency of iPSC induction. We are studying about the function of LIN28 during iPSC induction in detail.

Poster Board Number: 3196

SIRTUIN 1 IS INVOLVED IN THE GENERATION OF INDUCED PLURIPOTENT STEM CELLS

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Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSC) by forced-expression of 4 transcription factors. Recent studies show that the reprogramming efficiency can be improved by inclusion of small molecules that modulate epigenetic enzyme activities. We report here that sirtuin 1 (SIRT1), a member of the sirtuin family of NAD+-dependent protein deacetylases, is involved in iPSC formation. Our data indicated that Sirt1 protein levels in human and mouse embryonic stem cells (ESCs) decreased upon differentiation, while that of mouse fibroblasts increased during reprogramming. By using an efficient mouse fibroblasts reprogramming system using doxycycline (DOX) inducible Yamanaka's transcription factors delivered by piggyBac (PB) transposition, we further showed that Sirt1 was involved in reprogramming. Suppression of Sirt1 level decreased the efficiency of iPSC formation and increased the level of acetylated p53, a known substrate of Sirt1, during reprogramming. Conversely, resveratrol (RSV), an activator of Sirt1, increased the efficiency of iPSC formation. Consistently, the stimulatory activity of RSV on Sirt1 was associated with a reduction of acetylated p53. Furthermore, RSV and Sirt1 siRNA were applied at different periods during reprogramming. They were most effective in influencing the number of iPSC colonies formation in the initiation phase of reprogramming.

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The results in this study provide support on the possible use of epigenetic regulatory molecules in improving the efficiency of iPSC production.

Poster Board Number: 3198

BUTYLIDENEPHTHALIDE MAINTAINED THE PLURIPOTENCY OF INDUCED PLURIPOTENT STEM CELLS BY ACTIVATING JAK/STAT3 PATHWAY

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Induced pluripotent stem (iPS) cells were generated from somatic cells via introducing of four transcriptional factors: Oct4, Sox2, c-Myc and Klf4 by virus system in 2006. Although there were many ways to generate iPS cells, low efficiency was still a big hurdle need to overcome. In addition, as same as embryonic stem (ES) cells, iPS cells need to maintain the pluripotency by treating with leukemia induced factor (LIF), an expansive reagent. The aim of this study was to find the pure compound extracted from traditional Chinese medicine that not only could maintain the pluripotency in the ES cells and iPS cells, but also could increase the efficiency of iPS cells generation in the future. In this study, we showed that the gene expression levels of Oct4 and Sox2 in MEF cells up-regulated after treated with 10ug/ml N-Butylidenephthalide (BP), the extract from Angelica sinensis. Interesting, this effect was lower when the concentration higher (20ug/ml and 40ug/ml). We used the MTT assay to test whether these concentrations made the cell apoptosis and this data shown not. We also used iPS cells treated with different concentration of BP (replaced LIF in the culture medium) to test whether it was useful to maintain the pluripotency of iPS cells. The data showed that the expression levels of several stem cell markers in BP-treated iPS cells higher than LIF-out control, including alkaline phosphatase, SSEA1 and Nanog. This data determined that BP could maintain the pluripotency of iPS cells. In addition, we used the microarray analysis to find the major BP regulatory pathway. Microarray analysis showed that the top three deregulated pathway were PPAR, ECM and Jak-STAT pathway. We determined that phospholation-JAK2 and phospholation-STAT3 protein level was increase after BP treatment. In conclusion, our data demonstrated that BP could maintain the pluripotency of iPS cells though the Jak-STAT pathway. BP will become a health food to activate the stem cells. It is also useful to increase the efficiency to generate the novel iPS cells in the future.

Poster Board Number: 3200

POTENTIAL ROLE OF TCL1A IN THE PLURIPOTENT REPROGRAMMING OF FIBROBLASTS

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Induced pluripotent stem cells (iPS) derivation is an inefficient process and the reprogramming mechanisms are largely unknown. The main reason of the difficulty is that iPS phenomenon may occur in a stochastic manner. As iPS might be similar to embryonic stem cells (ESC) and offer novel therapeutic strategy to generate patient-specific stem cell lines, it would be important to elucidate the mechanisms and to improve the efficiency. With this in mind, the aim of this study was to analyze the global expression profile of TSM, a reprogrammed human fibroblast generated with forced expression of TCL1A, SOX2 and C-MYC, and to compare with the donor fibroblast cell line and with ESCs in order to identify genes that could modulate the reprogramming process. Moreover, we established cell lines with overexpression of TCL1A individually in order to find out its contribution to the reprogramming process, as this transcription factor had never been used for reprogramming before. We used a microarray platform composed by 44,000 human genes to perform the gene expression profile. We further validated some gene transcripts levels by quantitative PCR (qPCR). First, we investigated if the fibroblast specific genes were correctly silenced in the TSM cells, since this is the first step in the reprogramming process. To illustrate the change toward an embryonic stem-like transcriptome, using bioinformatic tools we selected a group of fibroblast signature genes and analyzed their microarray expression in the TSM cells. The results showed that the introduction of TCL1A, C-MYC and SOX2 into the TSM cells successfully silenced the fibroblast specific genes. THY1 and PLAU genes, which are markers of fibroblast lineage, were down-regulated in TSM cells compare to the donor fibroblast. Then, we examined genes involved with the mesenchymal-epithelial transition (MET), since this process had been suggested to be critical in the initial of mouse iPS derivation. We searched for 44 genes in the microarray libraries and found that these genes had been modulated after the induction and have a similar pattern of expression to the ESCs. We identified that important epithelial markers were activated in the TSM cells, including CDH1. Moreover, COL1A2 and CDH2, which are mesenchymal markers, were downregulated in the TSM cells. We also detected that SNAI1, TWIST1, and TGFB1, that are inhibitors of MET, were downmodulated in TSM cells. We next investigated if the pluripotent genes had been properly activated in TSM cells. We identified a core of pluripotent genes that had low expression levels compared to ESCs, such as POU5F1, NANOG and GDF3, indicating that they had not been correctly turned on. The qPCR results were in agreement with the microarray data. Finally, we analyzed the individual contribution of TCL1A, for which we expressed this gene in the fibroblast and compared with the donor cell and with ESCs. Intriguingly, qPCR analysis revealed that TCL1A alone was able to induce OCT4, LIN28, CDH1 and MMP9 mRNA expression and also to downregulate the MET repressors SNAI1, TWIST1 and TGFB1. In summary, the analysis revealed that the TSM cells presented an intermediary profile between the donor fibroblast and the ESCs, but the pattern of gene expression seems to move toward the embryonic-state. Moreover, TCL1A may contribute to the initial steps of human reprogramming through downmodulation of MET repressors.

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Poster Board Number: 3202

INDUCED PLURIPOTENT STEM CELLS DERIVED FROM TWO MESODERM CELL LINEAGES IMPART DIFFERENT EFFICIENCIES OF HEMATOPOIETIC STEM/PROGENITOR CELL GENERATION

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The ability to generate hematopoietic stem cells (HSC's) from patient derived induced pluripotent stem (iPS) cells, would enable the generation of an unlimited supply of HLA matched transplantable cells for the treatment of hematological disorders and malignancies. It has been proposed that the cellular starting material used to generate novel iPS cell lines imparts an epigenetic memory which in turn influences the differentiation capacity and efficiency of the iPS cells towards certain developmental lineages. Because the hematopoietic system is derived from the mesoderm germ layer during development, we decided to test the efficiency of blood cell generation from iPS cell lines derived from two human neonatal cell types with mesodermal origin; umbilical cord blood derived endothelial cells and amniotic fluid derived mesenchymal stem cells. With the ultimate goal of determining novel regulatory genes that can help direct ES/iPS cell differentiation towards blood lineages, we generated multiple iPS cell lines from the two cell sources, and evaluated the blood cell lineage differentiation capacity of the iPS lines using our state-of-the-art ES/iPS-to-blood differentiation protocol. Our optimized blood differentiation protocol allows for the generation of comparable numbers of hematopoietic progenitors from numerous iPS lines to those found in umbilical cord blood mononuclear cells isolates. From the 8 cord blood derived iPS lines we tested, the efficiency of hematopoietic cell generation ranged from 9.8 to 65.8% CD45+ hematopoietic cells (mean=34.6+/- 21.2% of which 5.8+/- 4% were CD45+CD34+ hematopoietic progenitors), while from the 9 amniotic fluid derived iPS lines, the efficiency ranged from 1.6 to 26.3% CD45+ hematopoietic cells (mean=14.2+/- 9% of which 2.9+/- 1.9% were CD45+CD34+ hematopoietic progenitors). The difference in the efficiency of hematopoietic cell generation between the cord blood and amniotic fluid derived iPS lines suggests that iPS reprogramming to a pluripotent state is not complete and that significant gene expression differences exist between the lines. Moreover, these differences can be determined and then exploited for more efficient blood cell production. To address these issues further investigation into the gene expression and epigenetic status of these lines is being performed.

Poster Board Number: 3204

PURIFIED MESENCHYMAL STEM CELLS ARE AN EFFICIENT CELL SOURCE FOR IPS CELLS INDUCTION

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Purpose: The iPS cell is ES like versatile cell induced by introducing several kinds of genes into somatic cells. It has been gained attention as a new cell source in the field of regenerative medicine. However, the induction efficiency was very low, and it is uncertain that which cell type should be responsible for the iPS cells induction. In addition, it has been known that it was difficult to produce germ-line competent iPS cells from adult tisuue. The purpose of this study is, therefore, to establish a higher quality of iPS cell from adult tissue. The cell source for the iPS induction is mainly fibroblast that represents heterogeneous population. Therefore, we assumed a possibility that only a very small amount of stem cell included in mixed population acquired the pluripotency by the gene introduction. Then we hypothesized that reprogramming efficiency of the stem cell is quicker than that of the differentiating cells. In this project, we performed comparative study of the induction efficiency by using adult mouse tail tip fibroblast (TTF), Osteoblast (OB) and high purity mesenchymal stem cells (MSCs). Method: We isolated enriched fraction of MSCs (PαS: CD45-/Ter119-/PDGFRa+/ Sca-1+ fraction) based on the specific cell surface markers expression by flowcytomery from Nanog-EGFP-IRES-Puror mouse. PαS represents 120,000 fold enriched fraction of MSCs compared with the conventional adhesion culture separation. The three or four Yamanaka factors were induced into PαS cells. We examined the introduction efficiency and the quality by comparing the numbers of colony formation and the gene expression, etc. Result: Compare to OB and TTF, the numbers of PαS derived iPS colonies were significantly increased. When five GFP+ colonies were picked from each cell sources randomly and analyzed with RT-PCR, all PαS-iPS clones expressed most ES cell marker genes. Silencing of the transgene was also observed with all PαS-iPS clones. DNA microarray analyses confirmed that PαS-iPS cells had ES cell like gene expressions. Compare to OB and TTF, PαS cells improved reprogramming efficiency and iPS quality. Moreover, iPS cells from PαS cells generated similar ratio of germ-line chimera as from ES. Germline-competent iPS cells express spermatogenesis related genes from DNA microarray analysis.

Poster Board Number: 3206

TRANSPOSON BASED SCREEN TO IDENTIFY OCT4 REPLACEMENTS IN REPROGRAMMING

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Reprogramming of somatic cells to iPSCs involves the over-expression of four transcription factors, Oct4, c-Myc, Klf4 and Sox2. Although this phenomenon has been demonstrated to be reproducible in a spectrum of cell types and animal species, we are only beginning to elucidate the molecular mechanisms of this dedifferentiation process. Molecular pathways involved in chromatin remodelling and mesenchymal-to-epithelial transition have been suggested to play significant roles. Genetic analyses have revealed factors replacing or enhancing the initial defined set of four genes. Complementary to this, chemicals which are able to promote reprogramming have also been discovered. These pieces of information enable us take a mechanistic peek at the reprogramming process and provide clues to decipher the pathways behind the plasticity of cellular pluripotency. To identify additional factors, we plan to perform a genetic screen in mouse fibroblast cells to replace the function of Oct4 in the reprogramming process. We expect to uncover factors that could be (i) direct upstream regulators of Oct4, (ii) main downstream targets of Oct4 and (iii) participants of alternative pathways regulating gene targets of Oct4. These gene targets may shed light on the intricacies behind the molecular circuitry involving Oct4 and perhaps uncover novel pathways in the maintenance of pluripotency, hence deepening our understanding of the reprogramming process.

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Poster Board Number: 3208

ADIPOGENIC DIFFERENTIATION AND EXPRESSION OF INTEGRINS IN REPROGRAMMED NIH3T3 CELLS

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Introduction: Dedifferentiation of adult somatic cells into multipotent cells may provide an attractive source of stem cells for regenerative medicine. Interaction between the matrix proteins and integrin adjust many cells' function such as adhesion, migration, cell cycle and self renewal in stem cells. In the present study, NIH3T3 cells were de-differentiated by mouse embryonic stem cell (mESC) extract. The expression of pluripotency markers as well as α2, α5 and α6 integrin subunits were determined in reprogrammed NIH3T3 cells. Adipogenic differentiation was performed to determine the potency of reprogrammed NIH3T3 cells. Material & methods: NIH3T3 cells were cultured in DMEM medium and permeabilized with streptolycin O, then exposed to embryonic stem cell extract. To prepare the extract, mESCs were treated with lysis buffer containing protease inhibitor. After permeabilization, the reprogrammed cells were cultured on inactivated feeders and were passaged every 2 days until 10 days. To check the ESC markers, enzyme assay, immunocytochemistry and RT-PCR were assessed by Alkalin phosphatase kit, Sox2 and Nanog antibodies, and Oct3/4 primer respectively. To assess the expression of integrins in reprogrammed cells, RT-PCR techniques was done for α2, α5 and α6 primers. The adipogenic differentiation was performed to determine the potency of reprogrammed NIH3T3 cells. Cell aggregates from the third passage were removed and cultured in adipogenic medium for 12 days, then were stained with Oil red. Results: NIH3T3 cells treated with mESC extracts showed noticeable changes in cell morphology as early as day 2 post-treatment forming colonies similar to typical mESC morphology by day 8, after three passages. The results of Alkaline phosphatase assay and immunocytochemistry staining indicated that these colonies showed the alkaline phosphatase activity and they expressed Sox2 and Nanog antibodies. RT-PCR revealed that the colonies also express Oct3/4. NIH3T3 cells, ESCs and reprogrammed cells expressed α2 integrin. α5 integrin expression was greatest in reprogrammed cells followed by the expression of this integrin in NIH3T3 which in turn was more than in ESCs. α6A integrin was expressed in NIH3T3 cells while α6B integrin was expressed in ESCs and in very low quantity was expressed in reprogrammed cells. The reprogrammed NIH3T3-derived EB like structures were differentiated into adipocytes by culturing in appropriate induction media. The intercellular lipid vacuoles and adipose droplet were stained with oil red. Concusion: These data provide evidence for the generation of ES like cells from differentiated somatic cells by mESC extract also expression profile of integrins after de-differentiation by ESC extract. Cytoplasmic lipid accumulation and the expression of oil red show the potency of reprogrammed NIH3T3 into different cell types. Our observations demonstrating that mESC protein extracts can reprogram somatic cells toward multipotency would argue that multipotent epigenome could be activated in somatic cells without fusion and forced expression of nucleic acids.

Poster Board Number: 3212

AN INDUCED PLURIPOTENT STEM CELL MODEL FOR LONG QT SYNDROME

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The recent breakthrough to generate patient-specific induced pluripotent stem cells (iPSCs) offers a new approach to model human disorders and could facilitate the development of novel therapeutic interventions. Long QT syndromes (LQTS) are genetic arrhythmogenic disorders characterized by abnormal ion channel function resulting in prolonged QT intervals on an electrocardiogram. A reduction of the rapidly activating delayed rectifier potassium channel current (IKr) caused by LQTS-associated mutations in the KCNH2/hERG gene can induce ventricular arrhythmia and cause sudden cardiac death. Here we report the development of disease-specific human iPSC lines from patients with type-2 LQTS caused by a splice site mutation in the KCNH2/hERG gene. The specific mutation disrupts the consensus sequence of the donor splice site of intron 9 and leads to the activation of a cryptic splice site 54 bp downstream. The cryptic splicing results in a full-length KCNH2/hERG protein with an insertion of 18 amino acids, causing a trafficking defect of the mutant channel. This was confirmed by KCNH2/hERG immunostaining in cardiomyocytes differentiated from established LQT2-iPSC lines, thereby providing the first trafficking defect studies with patient-specific iPSC lines. Electrophysiological measurements revealed prolonged action-potential-duration in LQT2 hiPSC lines when compared to healthy control lines. Ongoing single-cell patch clamp studies should confirm that the prolongation in action-potential-duration stems from a specific decrease in IKr. Furthermore, we are in the process of rescuing the phenotype in our LQT2-hIPSC lines by depleting the mutant KCHN2/hERG mRNA using short-hairpin RNA that targets the 54 bp insertion in combination with inducible overexpression of wild-type KCHN2/hERG. Our work demonstrates the opportunity of hiPSC technology to study human diseases in a dish, in our case, the abnormal functional phenotype of an inherited cardiac disorder.

Poster Board Number: 3214

TGF-β AND BMP SIGNALS LIMIT REPROGRAMMING OF AGING FIBROBLASTS FROM DYSTROPHIN-DEFICIENT MDX MUSCLE

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Duchenne muscular dystrophy (DMD) is a severe muscle wasting disorder. There is currently no effective therapy, but induced pluripotent stem cells (iPSCs) hold promise as a potential treatment for DMD. Since muscle biopsy is routinely performed to diagnose muscle dystrophy, cells derived from muscle tissue are a good candidate source for iPSCs generation. Furthermore, to determine the impact of the donor's age on reprogramming, we generated iPSCs from muscle derived fibroblasts (MuFs) of mdx mice aged 6 weeks, 6 months, and 14 months. MuFs from 14-month-old mdx mice showed lower proliferative activity and lower reprogramming efficiency, compared with those from younger mdx mice, and were refractory to full reprogramming by retroviral expression of Oct4, Klf4, Sox2, and c-Myc. Moreover, all iPSCs derived from 14-month-old mdx mice (14m-iPSCs)

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gradually lost Nanog expression, and regressed in conventional ES medium during passages. Interestingly, 14m-iPSCs exhibited higher expression levels of TGF-β family receptors (BMPR2, TGF-βR2, Acvr2a, Acvr1(ALK-2)) than embryonic stem cells or iPSCs derived from younger mice, and short-term treatment with inhibitors of TGF-β signaling (SB431542) or BMP signaling (Noggin) dramatically stabilized Nanog expression and promoted their self-renewal.

Poster Board Number: 3216

MOLECULAR INSIGHTS INTO INDUCED PLURIPOTENCY MEDIATED BY THE OCT4, SOX2, KLF4 AND C-MYC GENE REGULATORY NETWORK

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Induced pluripotent stem cells (iPS) can be derived from somatic cells by viral transduction of four defined transcription factors, including OCT4, SOX2, KLF4 and c-MYC (OSKM). Although previous studies have shown that epigenetic changes, suppression of p53, mesenchymal-to-epithelial transition (MET) and metabolic shift are key events for successful reprogramming, our understanding of cellular reprogramming is incomplete. Here, we have profiled genome-wide transcription levels using Illumina microarrays in the donor cells (human foreskin fibroblast-HFF1 cell line) and at three time points post OSKM transduction (24 h, 48 h, 72 h), as well as two iPS cell lines (iPS2, iPS4) and ES cell lines (H1, H9). We find that differentially regulated transcripts (relative to HFF1 cells), are enriched in genes associated with viral responses, as well as response to physiological oxygen, cellular morphological changes, apoptosis, cell proliferation, cell cycle and aging. Expression profiles suggest that full activation/depression of transcripts involved in epigenetic modifications, MET or metabolic shift had not occured within 72h of transduction. To find pathways associated with the induction and maintenance of pluripotency, we constructed a gene network from interactions available in the literature and identified relevant network paths within the context of regulated genes from the time series. Using this approach, we further describe molecular mechanisms involving GSK3B and FGF2 in the context of reprogramming.

Poster Board Number: 3218

THE ROLE OF DNMT3B IN REGULATING DNA METHYLATION PATTERNS DURING CELL REPROGRAMMING

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Immunodeficiency, Centromeric instability and Facial anomalies (ICF) syndrome is a recessive autosomal disorder linked to mutations in the DNA *de novo* methyltransferase DNMT3B. The loss of DNMT3B activity in cells from affected patients is closely associated with loss of DNA methylation at specific repeat regions and several gene promoters related to neurogenesis, development, and immune function. However, the roles of DNMT3B in regulating DNA methylation patterns during development and ICF pathogenesis remain to be fully understood. Using somatic cell reprogramming technology, we have generated iPSCs carrying DNMT3B mutation from fibroblasts of affected ICF syndrome patients. Next, we carried out unbiased genome-wide bisulfite sequencing to examine DNA methylation patterns in ICF fibroblasts and iPSCs. Our results show global demethylation in both CpG as well as CHG/CHH methylation sites in ICF iPSCs. Interestingly, while control and ICF fibroblasts show virtually no difference and minimal levels of CHG/CHH methylation, ICF iPSCs exhibited a significant hypomethylation in CHG/CHH methylation when compared to BJ control iPSCs. These results implicate that DNMT3B is partially involved in CHG/CHH methylation during somatic cell reprogramming. In addition, we have compared gene expression profiles in ICF fibroblasts and iPSCs. We found that ICF iPSCs exhibit much more deregulated genes when compared to fibroblasts, particularly in the category of embryonic development and morphogenesis, consistent with the important role for DNMT3B in pluripotent stem cells. The generation of ICF iPSCs will provide us great opportunities to investigate the pathogenic mechanisms of ICF Syndrome in development.

Poster Board Number: 3220

TAP63 AND ΔNP63 AFFECTS INDUCED PLURIPOTENT STEM CELL (IPSC) GENERATION THROUGH DICER, DGCR8 AND MIRNAS

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p63, along with p73 and p53, belongs to the extended p53 family. The three proteins share functional domains, including an N-terminal transactivation domain (22% identity), a central DNA-binding domain (60% identity) and a C-terminal oligomerization domain (35% identity), and have multiple isoforms due to alternative splicing and alternate promotor usage. Despite of the similarities in structure, these proteins have distinct functions in tumor suppression and development. The work of our laboratory and others supports a role for p53 in differentiation of embryonic stem cells and opposing stem cell formation during generation of induced pluripotent stem cells (iPSCs). p53-null and -mutant mouse embryonic fibroblasts (MEFs) have a significantly higher efficiency in iPSC generation. We chose to use this system to test whether p63 may likewise play roles in iPSC generation, and weather the specific isoforms of p63 exhibited similar or differing functions in this process. We used retroviruses carrying the genes of the four pluripotent factors (Oct4, Klf4, Sox2 and c-Myc) to infect primary MEFs and generate iPSCs. The full-length, trans-activating (TA) p63-/- MEFs show higher iPSCs generation efficiency compared to wild type MEFs, while the N-terminal deleted (ΔN) p63 MEFs showed lower iPSCs generation efficiency compared to wild type. This suggests that TAp63, like p53, might act as an anti-pluripotency factor, while ΔNp63 might act as a pluripotency promoting factor. Further analysis shows that the ΔNp63 level increased more than 10 folds during the process of iPSC generation in wild type MEFs. All together our data suggest that TAp63 and ΔNp63 might affect pluripotency by regulating miRNAs and/or cell cycle.

Poster Board Number: 3222

A PROPOSED PSYCHOSOCIAL EDUCATION AND TREATMENT SCHEME, NECESSARY AND SPECIFIC FOR FUTURE THERAPIES BASED ON INDUCED PLURIPOTENT STEM (IPS) CELLS.

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With the advent of findings and research regarding induced, pluripotent stem (iPS) cells, it is predictable that, in the near future, treatments for a variety of degenerative diseases will emerge based on this technology. Even

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with the wonderful results in animal models, the efficacy, lasting-potential, and acceptance of such new technologies is still undetermined. Within this space, there will be a bioethical need for patients and families to understand iPS cells, their purpose in treatment, and the social effect of, as yet, an undetermined range of outcomes. We propose the development of a scheme of social work interventions, including psycho-education within presenting informed consent and assisting clients in making an informed decision. In addition, we propose borrowing techniques applied to other medical diseases and treatments (Motivational Interviewing, Cognitive-Behavioral Therapy, etc.) to serve patients and their families in any given stage of action. This approach also would be essential for the medical community to acquire, sustain, and build a client base for iPS cell-based procedures. With the tailored application of interventions, clients and their families will be better able to accept and understand these new treatments, and to understand and honor their own personal feelings pre-, during, and post-procedures.

REGENERATION MECHANISMS

Poster Board Number: 3224

REGULATION OF OSTEOBLASTS AND OSTEOCLASTS BY HUMAN UMBILICAL CORD BLOOD DERIVED CD34 STEM CELLS IN OSTEOPOROTIC NOD/SCID MICE

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Osteoporosis is a diseased condition of bone developed due to low mineral density and loss of bone microarchitecture. A delicate balance of bone formation and bone degradation regulates the strength of bone. Osteoblasts and osteoclasts are two most important cells play critical roles in maintaining homeostasis of bone. Increased osteoclast activity and decreased osteoblast activity result in osteoporosis. Anabolic therapies and osteoinductive implants combined with change in lifestyle offer minimal improvements with complicated side effects. Stem cell therapies are being currently investigated for osteoporosis, however, their success is yet to be established. We have developed a nanofiber based ex-vivo expansion technology for the CD133+/CD34+ stem cells derived from human umbilical cord blood to provide essential number of functional therapeutic stem cells. To verify the in-vitro differentiation potential of nanofiber-expanded stem cell toward osteoblastic lineage, we induced differentiation using conditional media containing ascorbic acid and ß-glycerophosphate. To verify *in vivo* effectiveness, nanofiber-expanded stem cells were injected via intra-cardio ventricular route to osteoporotic animals (immunocompromised NOD/SCID mice) developed by injecting dexamethasone. After four weeks of stem cell injection, femurs were harvested and bone micro-architecture and bone remodelling was evaluated using micro-computed tomography (microCT), immunohistochemistry, morphometry and serum markers for bone remodeling. Bone marrow was also harvested from osteoporotic animals that received stem cell therapy and sham treated controls to evaluate osteoclast differentiation and functionality. Herein we show that nanofiber-expanded stem cells could be differentiated *in vitro* towards osteoblastic lineage. The osteoblastic differentiation was confirmed by using RT-PCR, immunocytochemistry, western blot and calcium specific staining (Alizarin Red S) for osteoblast related-gene and protein expressions. *In vivo*, stem cell therapy significantly improved bone mineral density, bone-remodeling and bone micro-architecture in osteoporotic mice compare to the sham treated controls evaluated by micro CT analyses, histomorphometry and *in vivo* immuno-labeling of bones. These improvements correlated with the elevated serum levels of osteocalcin, IL-1α, IL-10 and GM-CSF. Concurrently impaired differentiation and reduced activity of osteoclasts were observed in mice, which received stem cell therapy. Taken together, these data support an effective stem cell therapy for osteoporosis.

Poster Board Number: 3226

INFLUENCE OF HUMAN UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS (UCMSC) ON MONOCYTE/MACROPHAGE DIFFERENTIATION AND ACTIVATION IN MONOCYTE / UCMSC CO-CULTURES: IMPLICATIONS IN INJURY RESOLUTION

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Mesenchymal stem/stromal cells (MSCs) have been implicated to mediate regeneration and tissue repair in both *in vitro* and *in vivo* models of tissue/cell injury and inflammation. One of the initial events in an injury response is recruitment and differentiation of monocytes. Monocytes differentiated into cells of dendritic/macrophage lineage, facilitate maintenance of a microbe free environment and co-ordinate inflammatory responses at the sight of injury. Thus studying MSC interactions with monocytes is imperative to the understanding of how MSCs aid in tissue homeostasis post injury. In this study prototypic human monocytic cells lines, THP-1 and U937 and primary human monocytes have been activated with PMA to achieve monocyte differentiation and further activation was established in the presence of LPS and IFNγ. The influence of UCMSCs on monocyte differentiation and activation was studied in UCMSC-monocyte co-cultures or UCMSC macrophage co-cultures at different levels of activation or in presence of conditioned media obtained from proliferating UCMSCs. Monocyte differentiation and activation was scored through expression of surface markers CD14, CD11b, HLA-DR, co-stimulatory ligands and expression of scavenger receptors. Further phagocytosis capacity, cytokine release profiles, up-regulation of co-stimulatory molecules and oxidative status of the activated monocytes was studied to check the maturation status of activated monocytes in co-cultures with UCMSCs. Results indicate that though UCMSCs do not majorly influence monocyte differentiation per se it does skew the activation status of monocytes. Activated monocytes exposed to secretory factors from UCMSCs exhibit a more immature phenotype with higher phagocytosis capacity and reduction in Reactive nitrite intermediates (RNI). However, the antigen presentation capacity of macrophages cultured in the presence of UCMSCs is compromised thus indirectly controlling inflammation through attenuation of effector T cell responses. We thus speculate that UCMSCs might facilitate injury recovery by acting at monocyte activation at multiple levels without compromising on their anti-microbicidal functions but skewing them towards a more anti-inflammatory phenotype.

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Poster Board Number: 3228

THE EFFECT OF INTERLEUKIN (IL)-1β ON HUMAN ENDOMETRIAL AND ENDOMETRIOTIC STROMAL STEM/PROGENITOR CELLS

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The human endometrium is a highly regenerative tissue with remarkable proliferative and differentiation capacity. Endometrial stem/progenitor cells have been identified in human. Endometriosis is a common gynaecological disease affecting 5-10% of women. It is postulated to be related to the reflux of endometrial tissue during menstruation. Recently, clonogenic cells which exhibit somatic stem cell properties from ovarian endometriosis have also been reported. As chronic inflammatory responses can be found in patients with endometriosis, the effect of pro-inflammatory cytokine, interleukin (IL)-1β on endometrial and endometriotic stromal stem/progenitor cells was examined. Using functional clonogenic assay by seeding cells at very low density (500 cells/cm2), large colony forming units (CFUs) corresponding to colonies derived from stem/progenitor cells were observed. Treatment with IL-1β did not affect the formation of CFUs in normal endometrial stromal cells (NSCs, 0.048%±0.016%, n=16) and endometriotic stromal cells (ESCs, 0.062%±0.020%, n=16) when compared to the untreated controls. The effect of IL-1β on proliferative capacity of the clonogenic endometrial and endometriotic CFUs was examined by total cell output. With continuous treatment of IL-1β, the yield of a single CFU from NSCs (1.04±1.03x1016, n=3) was similar when compared to the control (7.52±6.62x1015, n=3). This suggests that IL-1β did not promote cellular senescence of endometrial stem/progenitor cells. Interestingly, the yield of a single CFU from IL-1β treated ESCs was about 30-fold higher than that of the control group (3.51±3.51x1010, n=3). There was significant difference (P<0.05) in the proliferative potential of individual CFUs of NSCs (1.04±1.03x1016) and ESCs (3.51±3.51x1010), demonstrating that microenvironment in which these stem/progenitor cells reside can influence their proliferation capacity. Expression of IL-1RI and IL-1RII mRNA was detected in all passaged cell types. It was assumed that each CFU was derived from 1 single stem/progenitor cell. The effect of IL-1β on self-renewal of ESCs was studied by subcloning (P1) of 5% of cells from individual primary (P0) IL-1β treated CFUs of ESCs (n=4) in an environment without the cytokine. Thus, a change in the number of CFUs in P1 would be due to the modulatory action IL-1β on self-renewal of clonogenic cells in P0. Results showed that cells from IL-1β treated CFUs produced more colonies (54.38±30.73) when compared to the untreated control (21.75±6.48) in P1. In summary, IL-1β does not affect the clonogenicity of NCCs. However, it increases the self-renewal capacity of ESCs, which may account for the increase in total cell yield per CFU. The results suggest differential response of NSCs and ESCs to IL-1β. Further studies will provide a better understanding on the aetiology of endometriosis.

Poster Board Number: 3230

REGULATORY MECHANISMS OF HUMAN ENDOMETRIAL STEM/PROGENITOR CELL-*IN VITRO* AND *IN VIVO*

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Human endometrium exhibits unique properties of cyclical regeneration and tissue breakdown throughout a woman's reproductive life. Within this highly regenerative tissue, it has been suggested that stem cells may be responsible for the cyclic growth of the endometrium. Human endometrial clonogenic cells as candidate somatic stem/progenitor cell have been identified, however little is known about the regulation of these cells. The aim of this study is to examine the key stem cell properties of human endometrial clonogenic cells *in vitro* and *in vivo*. Using the functional clonogenic assay, endometrial large (initiated by stem/progenitor cells) and small (initiated by transit amplifying cells) CFUs (colony forming units) were identified. The differentiation capacity of the large CFUs *in vivo* was studied by xenograft experiment. A mixture of endometrial epithelial/stromal cells derived from large CFUs was embedded in collagen I clots and transplanted beneath the kidney capsule of NOD-SCID (n=15). After 12 weeks, occasional proliferating cells (Ki67+) were detected within the xenograft. Smooth muscle-like structure (α-SMA+) and some newly formed vessels (VWF+) of human origin were also detected. The total cloning efficiency (CE) of epithelial cells was 0.06%±0.01% (n=13) at the proliferative phase and 0.09% ± 0.03% (n=9) at the secretory phase. The corresponding values for stromal cells was 0.27% ± 0.06%, (n=13) and 0.17% ± 0.06% (n=9), respectively. Individual large CFUs of epithelial and stromal cells had substantial self-renewal activity, being able to serial subclone 3.1 ± 0.1 (n=10) and 2.65 ± 0.3 (n=5) times, respectively. Whereas, small CFUs of both cell types could only self-renewal 2.0 times. After menses, the lower basalis give rise to the upper functionalis layer. During endometrial repair, we postulated that residual epithelial cells and stromal cells of the previous cycle stimulated the proliferation of endometrial stem/progenitor cells. Therefore, the importance of epithelial and stromal interaction was examined by a co-culture system using endometrial aspirate samples obtained on day 2 of the menstrual cycle. Epithelial and stromal cells were isolated from the aspirate and placed in a culture insert for coculture with epithelial cells or stromal cells collected from the basalis and seeded at cloning density. The total CE of endometrial stromal cells when co-cultured with day 2 epithelial cells was significantly higher (0.51% ±0.12 %, n=6) when compared to control (0.12% ± 0.05%, n=6, P<0.05). The increase was mainly due to the increase of small CFUs. The total CE of epithelial CFUs when co-culture with day 2 stromal cells (0.15% ± 0.06%, n=6) was similar to the control (0.17% ± 0.07%, n=6). In conclusion, the large CFUs are capable of differentiation into different lineages *in vivo*, supporting that their derivation from stem/progenitor cells. The clonogenic activity of endometrial cells was similar at different menstrual stages. Endometrial cells from day 2 of the menstrual cycle stimulated only the proliferation of the transit amplifying cells (small CFUs), indicating that the endometrial stem/progenitor cells (large CFUs) respond differently from its progenies. These findings contribute to a better understanding on the role of endometrial stem cells during the reproductive cycle.

Poster Board Number: 3232

IN VIVO BONE MARROW NICHE PROPENSITY REVEALS FUNCTIONAL HETEROGENEITY OF THE HUMAN HEMATOPOIETIC STEM CELL COMPARTMENT

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In vivo transplantation studies of prospectively isolated cells have uncovered significant phenotypic heterogeneity within the compartment of reconstituting human hematopoietic stem cells (HSCs). Despite the dependence on *in vivo* reconstitution of recipient bone marrow microenvironment, HSC heterogeneity has yet to be examined *in situ*. Here using an *in vivo* approach, we reveal that human NOD/SCID Repopulating Cells (SRCs) are heterogeneous for functional reconstituting ability. SRC in the trabecular bone area (TBA) of recipient immune deficient mice possessed superior self-renewal and regenerative capacity to those in long bone area (LBA). These niche features were consistent using mouse HSCs suggesting a conservation of this

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mode of HSC regulation. Molecular signatures of purified TBA-SRC uniquely indicated distinct Notch pathway regulation in contrast to SRC found in the LBA, and were physically identified to be exclusively associated with TBA bone endosteum expressing the Notch ligand Jagged-1. This *in vivo* niche affinity allowed independent *de novo* enrichment of human SRC based on direct Notch ligand binding. Our study demonstrates that heterogeneity of human HSCs can be functionally defined by the combination of specific BM niche occupancy and active *in situ* molecular interactions that define *in vivo* HSC capacity. This work was funded by a research grant from the Canadian Institutes of Health Research (CIHR).

Poster Board Number: 3234

IMPROVING LIVER REPOPULATION WITH MOUSE EMBRYONIC STEM CELL-DERIVED HEPATIC CELLS

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The shortage of liver donors is currently a major drawback hindering the treatment of liver diseased patients. One alternative to a whole liver transplantation protocol is the transplantation of hepatocytes. The ability of embryonic stem (ES) cell-derived hepatic cells to repopulate diseased livers remains inefficient. Published studies that generated ES cell-derived hepatic cells showed very little repopulation potential of those cells following transplantation in injured liver mouse models. Therefore our work focused on understanding the repopulation process in a step-wise manner and subsequently the identification of the limiting step to achieve successful transplantation with ES cell-derived hepatic cells. Fah-/-, Rag2-/-, IL2Rγ-/- (FRG) mice constitute an excellent model to follow diseased liver regeneration as the liver deficiency can be reverted by treating the FRG mice with a drug NTBC. Hepatic cells are transplanted while NTBC is removed to allow a growth advantage compared to the surrounding deficient hepatocytes. Using a reporter ES cell line expressing CD4 cDNA from the Foxa2 locus and CD25 cDNA from the Foxa3 locus (Foxa2-CD4, Foxa3-CD25), we have previously demonstrated an efficient and reproducible induction and hepatic specification of Foxa2+/Foxa3+ definitive endoderm population in the presence of BMP4 and ß-FGF. More recently, we demonstrated that mouse endothelial cells coordinate the hepatic specification of the Foxa2+/Foxa3+ definitive endoderm population through dual repression of Wnt and Notch signaling. Here we show that in a co-culture setting, human umbilical vein endothelial cells (Huvec) also highly induce Foxa2+/Foxa3+ definitive endoderm specification in the presence of Dkk1 (Wnt pathway inhibitor) and gamma secretase inhibitor (Notch pathway inhibitor) as mRNA levels of AFP and Albumin are increased 22-fold following 6 days of induction. We are now in the process of analyzing the regenerative ability of these hepatic cells using the FRG mice. However a growing literature indicated that the regenerative ability of ES cell-derived hepatic cells remains poor. Therefore, we will in parallel identify genes that are essential for successful liver repopulation by comparing the gene expression profile of Dlk+ hepatoblasts from embryonic 13.5 fetal livers (capable of successful repopulation) with that from ES cell-derived hepatic cells from day 12 of differentiation cultures. The gene signature characteristic of successful repopulation will be further discussed at the meeting. In parallel, these regeneration studies will be also performed using mouse iPS cell-derived hepatic cells. Our preliminary studies aimed to evaluate the relative ability to generate hepatic cells from mouse iPS lines compared with syngenic ES cell lines. Monitoring cell number and endoderm markers (cKit and CXCR4, ENDM1) expression by flow cytometry until day 6 of differentiation showed that iPS lines display a very similar proliferation rate, embryoid body morphology and endoderm emergence kinetic compared to the syngenic ES cell line. iPS cells and ES cells display a similar functional capacity to form definitive endoderm. Altogether, this study will define the limiting step of liver regeneration with ES cell-derived hepatic cells and identify key gene candidates for successful liver regeneration process.

Poster Board Number: 3236

SYSTEMIC ADMINISTRATION OF MULTIPOTENT MESENCHYMAL STROMAL CELLS INTO TYPE 1 DIABETIC MICE RESTORES THE BALANCE BETWEEN ANTI-INFLAMMATORY AND PROINFLAMMATORY CELLS, ALLOWING THE REGENERATION OF PANCREATIC ISLETS

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Type 1 diabetes mellitus (T1D) results from a cell-mediated autoimmune destruction of pancreatic beta-cells. Any available treatment allows patient cure. Previously, we demonstrated that the intravenous administration of a single dose of bone marrow-derived multipotent mesenchymal stromal cells (MSC) into mice with T1D reverted hyperglycemia, reduced glycated-hemoglobin level and increased insulinemia. This phenotypic reversion correlated with the recovery of the number, morphology and function of pancreatic islets. Our next aim was to evaluate whether transplanted MSC modify the imbalance between anti-inflammatory and proinflammatory cells characteristic of individuals with T1D. By flow cytometry and immunohistofluorescence, we studied the biodistribution of donor MSC in mice with T1D. Donor cells were isolated from isogenic mice that express GFP (MSCGFP) and intravenously administered into mice with T1D. Seven days post-administration, MSCGFP were mainly found in Peyer's patches and in minor degree in inguinal and pancreatic lymph nodes and bone marrow. Sixty-five days post-administration, MSCGFP were present almost exclusively in Peyer´s patches. At days 7 and 65, transplanted MSC were scarcely detected in the pancreas parenchyma. By flow cytometry, we determined the abundance of regulatory (CD4, CD25, Foxp3) and autoreactive (CD4low, CD40high) T cells in blood, spleen, bone marrow and pancreatic lymph nodes of mice with T1D transplanted or not with MSC. Seven and 65 days post-administration, in MSC-treated mice we found more regulatory and less autoreactive T cells, when compared with untreated mice. Furthermore, we observed that T cells isolated from MSC-treated mice were less prone to produce proinflammatory cytokines [IL2, INFgamma, TNFalpha] compared with cells isolated from untreated mice. By qRT-PCR, we assessed the expression level of anti-inflammatory/cytoprotective [IL4, IL5, IL10 and IL13] and proinflammatory [IL1beta, IL18, TNFalpha, TGFbeta, INFgamma, MCP1 and ICAM1] markers in the pancreas of mice with T1D transplanted or not with MSC. At day 7 post-administration, in the MSC-treated mice IL1beta, IL18, TNFalpha and MCP1 were expressed at basal levels, while in untreated mice all proinflamatory markers were overexpressed. At day 65, IL4, IL5 and IL13 were expressed at basal levels in the pancreas of MSC-treated diabetic mice, while in untreated mice those cytoprotective factors were downregulated. Our results show that in mice with T1D, MSC administered systemically mainly home into secondary lymphoid organs, induce the generation of regulatory T cells, reduce the number and the activity of autoreactive T cells and recover the expression of pancreatic cytoprotective factors. Hence, the transplantation of MSC restores the balance between anti-inflammatory and proinflammatory cells, resulting in a decline of the destruction of pancreatic beta-cells newly generated from endogenous progenitors.
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Poster Board Number: 3238

MOUSE KIDNEY PROGENITOR CELLS ACCELERATE RENAL REGENERATION VIA DIFFERENTIATION-INDEPENDENT PATHWAY

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Acute tubular necrosis is followed by regeneration of damaged renal tubular epithelial cells. Renal progenitor cells are supposed to be one of the sources. The purpose of the study is to unravel the possible mechanism of how renal progenitor cells accelerate renal regeneration after injury. A unique population of cells that behave in a manner consistent with renal progenitor cells was isolated from Myh9 targeted mutant mice, namely mouse kidney progenitor cells. Mouse kidney progenitor cells rescued renal damage as manifested decreases in peak serum urea nitrogen and the infarct zone when these cells were injected into the kidney of SCID mice after ischemic injury. By flow analyses, GFP-positive cells of the kidney increased from 3.4% on day 2 to 6.7% on day 30 after injury. It is interesting that conditioned medium of renal progenitor cells also rescued renal damage. *In vitro*, renal progenitor cells as well as conditioned medium not only accelerated primary proximal tubular cells proliferation but also decreased apoptosis after ischemic injury. Our results indicate that renal progenitor cells might accelerate renal regeneration after ischemic injury via paracrine effects.

Poster Board Number: 3242

ALLOGRAFT OF MOUSE UMBILICAL CORD-DERIVED MESENCHYMAL STEM CELLS INTO ISCHEMIC BRAIN ENHANCED THE GENERATION OF NEURAL CELLS FROM BONE MARROW- AND SVZ-DERIVED CELLS

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Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) have recently been identified as a promising candidate for cell-based therapy in various preclinical experimental models, such as Parkinson's disease, cerebral ischemia, spinal cord injury, and liver fibrosis. In addition, the induced pluripotent stem cells from hUC-MSCs have been successfully generated and shown higher efficiency than induced from fibroblasts (<0.01% vs. 0.4%). However, there is a growing concern on rejection and alteration of genetic code of this xenotransplant approach. In this study, a novel mouse UC-MSCs (mUC-MSCs) derived from wild-type and GFP transgenic mice have been successfully isolated, expanded, characterized, and differentiated into cells that distinct from its origin *in vitro*. We have administrated mUC-MSCs in several animal models, including hematopoietic reconstitution, murine focal cerebral ischemia, and acute liver failure, to explore their therapeutic effects. Our results indicated that post-irradiated intravenous infusion of mUC-MSCs could increase survival rate of γ-irradiated mice. And mUC-MSCs can differentiate into hematopoietic lineage cells in peripheral blood. Intracerebral administration of mUC-MSCs into ischemic brain significantly reduced infarct volume (63% vs. 47%), improved neurological behavior (a 1.6-fold increase in gait swing speed), and increased homing of BM-derived cells to ischemic cortex by up-regulation of SDF-1α. These BM-derived GFP+ cells were co-localizated with markers for microglia, endothelial cells, and to a less extend neuronal cells, but not astrocytes. Furthermore, semiquantitative RT-PCR revealed that mUC-MSCs expressed the mRNA of VEGF, GDNF, and SDF-1, but not FGF2 and BDNF. Intriguingly, hUC-MSCs do express VEGF, GDNF, BDNF, but not SDF-1. In summary, allograft of mUC-MSCs into various murine disease models might shed more light on the mechanism underlying the beneficial effects of cell therapy.

Poster Board Number: 3244

UMBILICAL CORD BLOOD DERIVED ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS CAN REPAIR HIND LIMB ISCHEMIA IN MICE.

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Peripheral vascular disease (PVD) is a condition that affects the vasculature outside of the heart and brain, causing an acute or chronic ischemia. It is also commonly referred to as peripheral arterial disease (PAD). The risk of suffering from PVD is significantly higher in diabetic patients and is a major complication of type 2 diabetes. While it is seen in 15% of the general population over 55 years old, the risk is increased up to four times in diabetic patients. Currently there are only two approaches for PVD management, surgery and medication. Therapies to improve blood flow, which include bypass surgery, stent or angioplasty, only target larger vessels and are not generally applicable for smaller vessels. The lack of proper treatment can result in amputation of the affected limb. Current treatments target slowing the progress of the disease but our aim is to reverse tissue damage, improve blood flow and repair damaged muscle. We have previously demonstrated that CD45+ human umbilical cord blood cells or bone marrow cells are capable of generating non-blood cell types after culture in FGF4, SCF and Flt3-ligand. When the post cultured cells are injected into the ischemic muscle they engraft and differentiate into new blood vessels as well as contribute to the enlargement of existing vessels. Sections were stained for human muscle actin and CD31 to identify human smooth muscle actin and endothelial cells respectively. The animals that received cells had high levels of vasculo- and arteriogenesis and a 75% normal return of blood flow. Our cells, referred to as multi-potential stem cells (MPSC) have proven to have higher levels of engraftment and repair than current products in clinical development (CD34+ cells).

Poster Board Number: 3246

MOUSE GERM PLASM-LIKE DOT CELLS TRANSFORM INTO EPITHELIAL AND HAIR FOLLICLE STEM CELLS IN TISSUE REGENERATION.

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Recent evidences suggest that skin epithelial stem cells are tissue specific. Therefore, we asked if there is a pre-stem cell population that gives rise to all cell types including epithelial cells. We have found, in the mouse blood, a group of E-cadherin-positive bodies (germ plasm-like Dot cells) that have strong regenerative activities. Immuno-electron microscopy confirms that these small bodies are not eukaryotic cells. They are about 1 μm, contain nuclear acid, and do not have nuclear membrane or intracellular organelles. Germ plasm-like Dot cells transform into small stem cells through a process including aggregation, fusion, and nuclear programming when they are in eukaryotic microenvironments. Results from *in vivo* transplantation demonstrate that germ plasm-like Dot cells-derived stem cells regenerate interfollicular epithelium via mesenchymal-epidermal transition; germ plasm-like Dot cells-derived hair bulb stem cells regenerate entire hair follicles. Our data provide evidence that germ plasm-like Dot cells transform into skin specific stem cells, which further divide symmetrically to regenerate different types of skin structures.

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Poster Board Number: 3248

ENHANCED RESISTANCE OF EPCS DERIVED FROM HESC AGAINST OXIDATIVE STRESS THAN CB EPCS DUE TO UPREGULATED ANTIOXIDANT MOLECULE EXPRESSION

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Endothelial progenitor cells (EPCs), which can obtained from umbilical cord, peripheral blood, bone marrow and placenta play an important role in tissue regeneration and vascular repair, but there still have obstructions, including limited proliferative capacity and low engraft efficiency, to use in clinical field. As these reasons, we derived EPC like cell population from hESCs (hESC-EPCs), to overcome therapeutic cellular source limitation, on the basis of unlimited proliferation capacity and pluripotency of hESCs. In this study, hESC-EPCs were sorted by endothelial cell marker CD133+/ KDR+, and we confirmed that hESC-EPCs were functionally equal to cord blood derived EPCs (CB-EPCs) by characterization test through RT-PCR, FACs analysis, ac-LDL uptake and tubule forming assay, *in vitro*. Also, to define fundamental characteristics of hESC-EPCs at molecular level, we performed proteomic analysis using 2-DE and LC-M/MS protein identification method. As a result, specific up-regulated protein expression profile, which involved in vascular structure reconstruction, anti-oxidant/ anti-apoptosis and migration, were verified in hESC-EPCs. To investigate whether anti-oxidant related proteins, which up-regulated in hESC-EPCs, are functionally contributed on defense mechanism against oxidative stress, we compared anti-oxidant ability of hESC-EPCs and CB-EPCs using *in vitro* wound healing assay, hypoxia culture and H2O2 treatment. Consistently with proteomic result, up-regulated expression pattern was sustained and hESC-EPCs were shown more resistant in oxidative stress. More interestingly, anti-oxidant related proteins expressed in hESC-EPCs were more up-regulated in oxidative stressful condition than normal culture condition. Also, in hindlimb ischemic disease model mice, the therapeutic effect of hESC-EPCs also confirmed. As same as our expectation, up-regulated antioxidant molecule expression in hESC-EPCs was equipped with an antioxidative defense, to provide resistance against oxidative stress than CB-EPCs. Taken together, the data shown in this study suggested that hESC-EPCs will be more optimized cellular source on several ischemic diseases due to their resistant effect on oxidative stress and this combinational approach using stem cell and proteomics could give us clue to solve therapeutic mechanism.

Poster Board Number: 3250

ORIGINS OF THE NUCLEUS PULPOSUS: IMPLICATIONS FOR STEM CELL THERAPY OF DEVELOPMENTAL DISC DISEASE.

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Background: Low back pain is the most common musculoskeletal problem with a lifetime prevalence of 82%. A significant proportion of lower back pain is attributed to degeneration of the intervertebral disc (IVD) and more specifically the nucleus pulposus. To reverse the pathological process of disc degeneration, cell viability and cellular phenotype in the nucleus pulposus should be targeted; an approach that requires first characterization of the developmental origins of cell types within the nucleus pulposus.
Methods: We have generated a novel notochord-specific Cre knock-in mouse in which Cre recombinase has been targeted to the Noto locus, a gene that is expressed exclusively in the node and developing notochord. Upon mating the NotoCre/+ mouse with a ROSA26 conditional LacZ reporter mouse, we have performed lineage-tracing experiments throughout development. Results: Fate mapping studies have shown the notochord gives rise to the nucleus pulposus in the mature mouse. This is achieved by β-galactosidase staining, which is present throughout the majority of the spinal column. Cellular localization of β-galactosidase demonstrates that both notochord cells and chondrocyte-like are derived from the embryonic notochord. This indicates that the notochord exclusively gives rise to the nucleus pulposus and that notochord cells act as embryonic predecessors of all the cells found within the nucleus pulposus. Conclusions: The ultimate goal of this study was to determine the fate and function of notochord cells during spinal skeletogenesis. These experiments show that nucleus pulposus is exclusively derived from the notochord and that all cells within are derived of the embryonic notochord. This study provides evidence that notochord cells are tissue specific stem cells for the nucleus pulposus and therefore has the potential for future tissue engineering strategies.

Poster Board Number: 3252

PLEIOTROPHIN IS A NICHE FACTOR WHICH REGULATES ENGRAFTMENT OF HEMATOPOIETIC STEM CELLS

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Hematopoietc stem cells (HSC) are maintained at a quiescent state during steady-state hematopiesis. HSC are charachterized by their ability to respond quickly to hematopoietic stress *in vivo*, as well as in culture, by increasing proliferation and inducing early lineage commitment. It is likely that secreted niche factors are involved in this transition from quiscence towards activation. We here show that knockdown of the secreted factor pleiotrophin (Ptn-KD) in stromal cells increases proliferation as well as production of hematopoietic progenitors as well as HSC activity in co-cultures with lineage-negative (Lin-) hematopoietic cells. Moreover, engraftment of cells co cultured with PtnKD stromal cells is associated with increased numbers of Cd34- Lin- Sca+ Kit+ (LSK) cells and dominant myeloid regeneration. Despite clear effects of Ptn deficiency in co-cultures, steady-state hematopoiesis is not altered in Ptn knockout (Ptn-/-) mice. This suggests that Ptn May be involved in regulating HSC quiscence. Indeed, engraftment of wild-type HSC in lethally irradiated Ptn-/- mice mirrors the cultures on PtnKD stromal cells in that engraftment is increased in serial transplantations with progressive myeloid skewing and accumulation of CD34- LSK donor cells. On a molecular level, steady state Ptn-/- LSK cells express decreased levels of cyclin D1, but an increased expression of the myeloid master-regulator C/EBPα. In contrast, the observed increase in hematopoietic regeneration in co-cultures on PtnKD stromal cells is associated with UPregulation of cyclin D1 (Ccnd1), whereas again C/EBPα was also increased. Thus, the difference between steady-state quiscence and activated engraftment appears to lie in strict regulation of cyclin D1 through Ptn. Since neither in steady state Ptn-/- cells, nor in wild-type LSK cells cultured on PtnKD stroma, the transcript or protein levels of β-catenin were influenced. The regulation of cyclin D1 through Ptn appears to be independent of canonical Wnt signaling. In conclusion, our results point to different regulatory mechanisms in normal hemostasis and in hematopoietic regeneration. Moreover, our results support the idea that microenvironmental Ptn mainly regulates cyclin D1-dependent hematopoietic regeneration.

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Poster Board Number: 3254

HEDGEHOG/WNT FEEDBACK SUPPORTS REGENERATIVE PROLIFERATION OF EPITHELIAL STEM CELLS IN BLADDER

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Epithelial integrity in metazoan organs is maintained through the regulated proliferation and differentiation of organ-specific stem and progenitor cells. Although the epithelia of organs such as the intestine regenerate constantly and thus remain continuously proliferative, other organs, such as the mammalian urinary bladder, shift from near-quiescence to a highly proliferative state in response to epithelial injury. The cellular and molecular mechanisms underlying this injury-induced mode of regenerative response are poorly defined. Here we show that the proliferative response to bacterial infection or chemical injury within the bladder is regulated by signal feedback between basal cells of the urothelium and the stromal cells that underlie them. We demonstrate that these basal cells include stem cells capable of regenerating all cell types within the urothelium, and are marked by expression of the secreted protein signal, Sonic hedgehog (Shh). Upon injury, Shh expression in these basal cells increases and elicits increased stromal expression of Wnt protein signals, which in turn stimulate the proliferation of both urothelial and stromal cells. The heightened activity of this signal feedback circuit and the associated increase in cell proliferation appear to be required for restoration of urothelial function and, in the case of bacterial injury, may help clear and prevent further spread of infection. Our findings provide a conceptual framework for injury-induced epithelial regeneration in endodermal organs, and may provide a basis for understanding the roles of signaling pathways in cancer growth and metastasis.

Poster Board Number: 3256

INHIBITION OF CXCR4 RECEPTOR ENDOCYTOSIS INDUCES CXCR4 SURFACE EXPRESSION AND ENHANCES MIGRATION OF FETAL MESENCHYMAL STEM/STROMAL CELLS

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Both adult and fetal mesenchymal stem/stromal cells (MSC) preferentially home to sites of tissue injury but the underlying processes are poorly understood, limiting ability to target MSC to regions of tissue damage. The CXCR4-SDF-1 axis is active in MSC, which migrate toward SDF-1 *in vitro*, but has not been studied in fetal MSC, which have translationally advantageous properties over adult MSC. Only a subset of adult MSC express surface CXCR4, and up-regulation of CXCR4 by viral transduction increases MSC homing to injury sites. CXCR4 like other G-protein receptors undergoes both ligand-dependent & ligand-independent endocytosis, and its trafficking in turn should determine the outcome of CXCR4 signalling. We investigated surface & cytoplasmic expression of CXCR4 on human fetal bone marrow MSC. As in adult MSC, CXCR4 surface expression in fMSC is low (3-4%), with most CXCR4 protein sequestered intracellularly (>90%). Using immunofluorescence microscopy, we showed that CXCR4 is colocalized with Rab5 & Rab11, markers associated with early & recycling endosomes respectively. CXCR4 also colocalized albeit to a lesser extent with the lysosomal marker, Lamp-1. Using a neutralizing antibody against SDF-1, we demonstrate that endocytosis of CXCR4 is independent of endogenously produced SDF-1. Treatment of fMSC with blebbistatin & dynasore, inhibitors of endocytosis, increased the surface expression of CXCR4 more than 5 fold (untreated mean fluorescence intensity 3.8±0.25 vs blebbistatin 33.4±11.3 dynasore 25.6±5.4 respectively, $p<0.05$) relative to untreated cells. Migration of fMSC to SDF-1 was increased by both inhibitors in *in vitro* chemotaxis assays (blebbistatin 2.8±0.21, dynasore 2.6±0.22, fold more than untreated cells, $p<0.01$). These data implicate constitutive endocytosis in the regulation of CXCR4 membrane expression on fetal MSC, & suggest pharmacological strategies to enhance migration of systemically-transplanted cells. Improving homing would be advantageous not only to increase the therapeutic effect but also to reduce potential harm posed by inappropriate uptake of MSC in healthy tissues.

Poster Board Number: 3258

BMP AND WNT PATHWAY REGULATION OF LINEAGE-SPECIFIC GENE PROGRAMS UNDERLIE HEMATOPOIETIC REGENERATION

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BMP and Wnt signaling pathways play key roles in hematopoietic development. Here we describe the effect of these pathways during stress-induced hematopoietic regeneration. Using heat-shock inducible transgenic fish lines that overexpress BMP2 or WNT8, we demonstrated accelerated marrow recovery following irradiation. Irradiation recovery was blunted by heat shock induced overexpression of the respective inhibitors Chordin and DKK1. Similar to the zebrafish regeneration results, competitive transplants with mouse bone marrow treated with the WNT agonist BIO led to enhanced chimerism. Inhibition of BMP diminished peripheral blood contribution in the presence of WNT stimulation, suggesting a conserved and cell intrinsic interaction for these signaling pathways in adult stress hematopoiesis. To examine potential target genes of these pathways that could account for the effects during irradiation recovery, we performed chromatin immunoprecipitation coupled with sequencing (ChIP-seq) with SMAD1 and TCF7L2, mediators of the BMP and Wnt pathways respectively, in erythrocytic and monocytic leukemias. Surprisingly, the Wnt and BMP co-localization in each cell type occurred at well-known lineage-specific genes associated with erythroid lineage in K562 cells, and with myeloid lineage in U937 cells. These data indicate cell type selective binding of TCF7L2 and SMAD1 at distinct hematopoietic genes according to cell lineage. Comparison of the ChIP-seq results with previously published GATA1/2 ChIP-seq results in K562 and C/EBPα in U937 showed that more than 70% of TCF7L2 and SMAD1 targets co-localize with these cell-specific factors. These data suggest selective binding of TCF7L2 and SMAD1 signaling-directed transcription factors in concert with cell specific master regulators at lineage distinctive genes. This specific binding occurs also in primary human hematopoietic progenitor cells (CD34+ cells) and in erythroid cells derived from CD34+ cells. A shift in binding of BMP and Wnt transcriptional regulators to erythroid specific genes upon directed differentiation was observed suggesting interplay between lineage restricted transcription factors and the signaling regulators. We demonstrated the biological significance of this type of interaction in flexed tail mutant mice with defective BMP dependent stress erythropoiesis due to a dominant negative mutation in SMAD5. In these mice, forced expression of lineage-specific transcription factors GATA2 or SCL restored the regeneration defect in SMAD-deficient erythroid progenitors. Our data support a model in which the BMP and Wnt pathways act on cell-specific gene targets and are guided by lineage-specific master regulators. Although dispensable for steady state hematopoiesis the BMP and Wnt pathways regulate tissue regeneration. Our data imply that an underlying mechanism of regeneration and self-renewal is the control of the entire hematopoietic program. The BMP and Wnt signaling transcription factors associate with master regulators of multiple hematopoietic cell types, providing a mecha-

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nism to stimulate the intrinsic hematopoietic program during differentiation and regeneration. The two first authors contributed equally to this work

Poster Board Number: 3260

CHANGES IN THE EXPRESSION OF MARKERS OF STEM /PROGENITOR CELLS LINKED TO FLUCTUATION IN LACTOTROPH POPULATION

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The pituitary gland shows dynamic adjustments in response to hormonal fluctuations such as puberty, pregnancy and lactation. The adaptation of this gland at different physiological requirements may be due to the recruitment and differentiation of stem/progenitor cells (S/P).These cells was located in the marginal zone that limits the pituitary cleft. In previous work we demonstrated that lactotroph population exhibit significant changes during lactation. The aim of this study was to identify pituitary S/P cells and quantify pluripotency markers: Sox2 and Oct-4, evaluating possible changes during lactation and estrous cycle. We used adult Wistar female rats in oestrus and diestrus II phase of estrous cycle and active lactancy. The pituitary gland were processed for ultrastructural immunocytochemistry, which allowed us to determine the topographic location of S/P cells by detection of differentiation markers: ß-catenin and vimentin at the subcellular level and to identify markers associated with S/P cell phenotype, the membrane receptor GFRa2 and transcription factors Sox2 and Oct-4. Morphometric quantification was carried out immunostaining of Sox2 and Oct-4 by electron microscopy using software Image J. Statistics: ANOVA-Fisher. Ciliated cells of the marginal zone were negative for ß-catenin, while vimentin positive cells were localized around those like as they were supporting cells. The GFRa2 receptor was expressed in cells that limit the pituitary cleft. The transcription factors Sox2 and Oct-4 is expressed in marginal zone cells with an increase ($p < 0.05$) during active lactation and oestrus phase compared to diestrus II phase of estrous cycle. These findings would seem to indicate the involvement of stem/progenitor cells in lactotroph population fluctuations on physiological conditions of high hormonal demand.

Poster Board Number: 3262

A BATF DEPENDENT DIFFERENTIATION CHECKPOINT LIMITS SELF RENEWAL AND FUNCTION OF HEMATOPOIETIC STEM CELLS IN RESPONSE TO DNA DAMAGE

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There is growing evidence that the accumulation of DNA damage and telomere dysfunction contributes to declines in stem cell function and tissue maintenance during aging and in response to chronic disease. Checkpoint responses that mediate the decline in stem cell function in response to DNA damage remain yet to be delineated. Here we conducted a functional genomic *in vivo* screen to identify genes impairing the function of hematopoietic stem cells in the context of chronic DNA damage induced by telomere dysfunction. Screening of an shRNA library of 1000 cancer related genes revealed that the shRNA mediated knock down of the transcription factor Batf was most potent to rescue the function of telomere dysfunctional stem cells. Specifically, Batf knockdown was 500fold enriched in telomere dysfunctional HSCs compared to wildtype HSCs when transplanted into lethally irradiated recipients. Our study shows that Batf is strongly upregulated in HSCs in response to telomere dysfunction or gamma-irradiation induced DNA damage and during mouse aging. Upregulation of Batf in HSCs in response to DNA damage was G-CSF/Stat3 dependent. shRNA-mediated knockdown of Batf rescued the repopulation capacity of telomere dysfunctional stem cells in transplanted recipients. This rescue in HSC function was associated with impaired induction of lymphocyte differentiation markers, cell cycle inhibitors (p53, p21, p16), and apoptosis in telomere dysfunctional or gamma-irradiated HSCs. Irradiation experiments revealed that lymphoid competent HSCs (CD150low CD34- KSL) were most sensitive to DNA damage induced stem cell depletion, which was rescued by shRNA mediated knockdown of Batf. Together, this study provides the first experimental evidence that DNA damage leads to induction of a Batf-dependent differentiation checkpoint limiting the repopulation capacity of HSCs and the self-renewal of lymphoid competent HSCs. It is conceivable that this molecular circuit contributes to the age-dependent skewing of HSC characterized by impairments in lymphopoiesis and increases myelopoiesis.

Poster Board Number: 3264

MODULATION OF THE LIN28/LET-7 PATHWAY ENHANCES WOUND HEALING AND REGENERATION.

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Planaria, zebrafish and newts are capable of epimorphic regeneration, the regrowth of anatomically complex organs after injury or amputation. In contrast, mammals lack the ability to regenerate injured limbs, hearts and spinal cords. In studies of the RNA-binding proteins Lin28a and Lin28b, which block the processing of let-7 microRNA family members to promote pluripotency in embryonic stem cells and to enhance somatic cell reprogramming, we have noted a surprising regeneration phenotype in several injury models. First, we found that Lin28a transgenic (Tg) mice have a markedly enhanced capacity to regrow hair after shaving in both 4 and 10 week old mice, reflecting rapid re-entry into anagen after a prolonged telogen phase. Second, Lin28a Tg mice show improved capacity for wound healing after 1 or 2mm ear hole punching, with decreased wound size at day 5, 8, and 25, and shorter time to full hole closure. In contrast, induction of let-7g in conditional transgenic mice delayed wound healing and resulted in incomplete wound closure. Third, Lin28a transgenic mice displayed enhanced soft tissue and bone regeneration after neonatal digit amputation, even when normalized for differences in animal size. Expression analysis in regenerating digits revealed upregulation of canonical let-7 target mRNAs including Lin28a, Hmga1/2, Igf2bp1-3, Igf1 and Igf1r. To determine if Lin28a Tg mice manifest enhanced regeneration via the same pathways as do MRL mice, a well-known regenerative strain, we performed epistasis experiments. Surprisingly, even in the MRL background Lin28a overexpression produced enhanced regeneration following digit amputation, suggesting non-overlapping, synergistic mechanisms of enhanced regeneration. We are currently exploring the cellular and molecular bases for these phenomena. In summary, we have demonstrated that mammalian regeneration and wound healing can be augmented by genetic modulation of the Lin28/let-7 pathway, which may be exploited therapeutically.

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REPROGRAMMING

Poster Board Number: 3266

DIRECT CONVERSION OF HUMAN FIBROBLASTS TO DOPAMINERGIC NEURONS

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Recent studies revealed that overexpression of defined factors enables direct conversion of somatic mouse cells into other mature cell types, such as cardiomyocytes, neurons or insulin-secreting beta cells. We show that the same three factors that convert mouse fibroblasts into neurons (Ascl1, Myt1l and Brn2) can be used to directly convert human fibroblasts into functional neurons, termed human induced neural (hiN) cells. The neuronal properties of the hiN cells were verified based on morphology, expression of neuronal markers (beta-III-tubulin, MAP2 and Synaptophysin), and electrophysiological characteristics of neurons. In the presence of the three conversion factors (Ascl1, Myt1l and Brn2), only few hiN cells expressed GABA or glutamate and no hiN expressed markers of dopaminergic or serotonergic neurons. We hypothesize that additional factors are needed to program hiN cells into subtype-specific neurons. Therefore, we performed direct neural conversion of human fibroblasts in the presence of two additional factors known to be important for dopamine fate specification. Under these conditions, hiN cells expressing TH, AADC and Nurr1 are formed. This study provides evidence that human fibroblasts can be directly converted into functional neurons. The ability to further direct the converted cells towards specific subtypes of neurons, exemplified here by dopamine neurons, offers exciting future possibilities to generate patient-specific neurons for disease modelling and brain repair.

Poster Board Number: 3268

DIRECT CONVERSION OF HUMAN SOMATIC CELLS INTO NEURONS BY DEFINED TRANSCRIPTION FACTORS

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Cell-lineage fates are considered stable once determined during embryonic development. However, experimental manipulations such as somatic cell nuclear transfer, cell fusion, or expression of lineage-specific factors can induce cell-fate changes in diverse somatic cell types. We recently observed that forced expression of a combination of three transcription factors, Brn2 (also known as Pou3f2), Ascl1, and Myt1l rapidly and efficiently convert mouse fibroblasts into functional neuron-like cells termed induced neuronal (iN) cells. Here, we asked whether iN cell reprogramming factors could also generate neurons from human cells. We show that a very similar approach yields iN cells from fetal or postnatal human fibroblasts. The human iN cells displayed typical complex neuronal morphologies, expressed an array of pan-neuronal as well as subtype-specific neuronal markers, and were stable after downregulation of the exogenous transcription factors. Importantly, the vast majority of human iN cells were able to generate action potentials, and many matured sufficiently to receive synaptic contacts from primary mouse cortical neurons. Our data provide a proof-of-principle that non-neural human somatic cells can be directly converted into neurons by lineage-determining transcription factors. We are currently optimizing the reprogramming factor combination and culture condition to improve the efficiency of generating functional human iN cells. These methods may allow robust generation of patient-specific human neurons for *in vitro* disease modeling or future applications in regenerative medicine.

Poster Board Number: 3270

DIRECT REPROGRAMMING OF HUMAN FIBROBLASTS INTO CARDIOMYOCYTE-LIKE CELLS BY DEFINED FACTORS

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Recently, we reported that mouse cardiac and dermal fibroblasts can be directly reprogrammed into cardiomyocyte-like cells by three defined transcription factors (e.g., Gata4, Mef2c, Tbx5), and we termed these cells induced cardiomyocytes (iCMs). 50% of cells in the human heart are fibroblasts, providing a vast pool of potential cells that could be reprogrammed into cardiomyocytes if an analogous cocktail of factors could be identified. In the current study, we found that a combination of seven defined transcription factors rapidly and efficiently reprogrammed human ES cell-derived fibroblasts or adult human dermal fibroblasts into cardiomyocyte-like cells. 30% of fibroblasts transduced with the human cardiac reprogramming factors expressed cardiac troponin T within 2-weeks. These cells expressed cardiac-specific markers and had a global gene expression profile similar to that of cardiomyocytes. These findings demonstrate that human fibroblasts can be directly reprogrammed into cardiomyocyte-like by defined factors. Reprogramming cardiomyocytes from patient-specific fibroblasts might provide a viable cell source for regenerative medicine and for modeling cardiac disease.

Poster Board Number: 3272

ELECTROPHYSIOLOGICAL PROPERTIES OF FUNCTIONAL NEURONS DIRECTLY CONVERTED FROM HUMAN FIBROBLASTS

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Recent reports have shown that mouse somatic cells can be directly converted to functional neurons using the combined expression of defined factors. We have now shown that the same strategy can be applied to human embryonic and postnatal fibroblasts by over-expression of the transcription factors Ascl1, Brn2, and Myt1l. Here we demonstrate using whole-cell patch-clamp recordings that the human fibroblasts are converted to functional neurons. These cells, at 30-32 days *in vitro*, exhibited passive and active membrane properties (e.g., input resistance, resting membrane potential, whole-cell currents, action potential threshold, amplitude, and duration) comparable to functional neurons. Depolarizing current injection elicited trains of TTX-sensitive action potentials. The converted cells could be directed towards a specific neuronal subtype when two genes involved in dopamine neuron generation, Lmx1a and FoxA2, were provided together with the three conversion factors. At 28 days *in vitro*, these cells showed passive and active membrane properties characteristic of mature neurons, and a proportion exhibited spontaneous action potentials and properties resembling those of midbrain dopamine neurons *in vitro*. Our results indicate that human embryonic and postnatal fibroblasts can be directly converted to functional neurons of specific subtypes.

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Poster Board Number: 3274

CENTRIOLE DEACETYLATION IS A CYTOPLASMIC EVENT VITAL FOR REPROGRAMMING HUMAN CELLS BY INDUCED PLURIPOTENCY

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Centriole Deacetylation is a Cytoplasmic Event Vital for Reprogramming Human Cells by Induced Pluripotency. Calvin Simerly1,2, Charles Easley IV1, Ahmi Ben-Yehudah1, Stacie Oliver1, Carrie J Redinger1, Dave McFarland1 and Gerald Schatten1,2,3. 1Division of Developmental and Regenerative Medicine, Department of Obstetrics, Gynecology, and Reproductive Sciences; 2Department of Cell Biology-Physiology; 3Department of Bioengineering; University of Pittsburgh, Pittsburgh, PA 15213, USA
Induced pluripotency (iPS) has revolutionized human stem cell biology since patient-specific, disease-bearing pluripotent cell lines can readily be generated for fundamental and translational investigations, and yet the underlying molecular mechanisms responsible for iPS have not been fully elucidated. While great strides have been made in understanding the roles of transcription factors and epigenetic chromatin modifications, our research focuses on the cytoplasmic mediators of reprogramming. Specifically, we have discovered that the microtubules which comprise the centrioles in the poles of the mitotic spindles of human pluripotent stem cells (hPSCs) are post-translationally modified. -Tubulin is acetylated at lysine 40 in centrioles found in human somatic and pluripotent stem cells (PSCs) at mitosis, with only weak localization in spindle pole microtubules. This centriole localization pattern persists in some early *in vitro* differentiation colonies and following cold depolymerization of microtubules. Interestingly, rescue of cold-treated PSCs demonstrates that the first dynamic microtubules to reassemble from the centrosomes are not acetylated. Between 30-60sec post-cold recovery in interphase cells, acetylated microtubule cables appear which are detached from the centrosome where dynamic microtubules are reassembling. Acetylated--tubulin is lost from centrioles after exposure to HDAC inhibitors (HDACi), especially inhibitors to HDAC6, which may well serve as a cytoplasmic Tubulin Deacetylase (TubDAC). We found that Tubastatin-A, Scriptaid, Trichostatin-A, and SAHA, but not Oxamflatin, induces a loss of centriole acetylation and a concomitant increase in spindle microtubule acetylation in human pluripotent hESCs and iPS cell lines. Likewise, HDACi treated cells exposed to cold followed by microtubule rescue for 2 min showed no centriole acetylation, but increased spindle microtubule acetylation. We also investigated if the HDAC6 inhibitor Tubastatin-A would prevent iPS reprogramming— Neither PSC colony formation nor of the PSC pluripotency marker expression (SSEA-4) are found. Tubastatin-A induces differentiation of hPSCs but without apparent effects on histone acetylation levels in pluripotent cells as well as in cells undergoing iPS reprogramming. These investigations focus on the role of the cytoplasm and cytoskeleton in mediating the events of nuclear reprogramming, and demonstrate tight cooperation between nuclear dynamics and cytoskeletal plasticity. In summary, this research demonstrates that post-translational modifications of microtubules, in addition to transcriptional regulation, are essential for both differentiation as well as reprogramming by iPS in human cells. We conclude that microtubule deacetylation in mitotic centrioles is required for reprogramming Human iPS cells. This then represents A Novel Cytoplasmic Example of 'Centriole Reprogramming.' Sponsored by the National Institutes of Health.

Poster Board Number: 3276

HUMAN IPS GENERATED WITH FUSION PROTEINS. FINE TUNING OF THE PROCESS WITH SMALL MOLECULES

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The possibility to directly reprogram somatic cells into iPS cells with recombinant proteins has been described from 2009 by several groups. Totally devoid of viral sequences, protein-based iPS might represent the ideal stem cell (SC) source for a number of applications, particularly in a therapeutic perspective. Generalization of this approach is linked to the optimization of a number of steps that include protein production and purification on one hand, and protein concentrations and timing of application on the other hand. An additional and crucial parameter is the application of small molecules to improve the reprogramming process. The coding sequences of human OCT4, NANOG, KLF4, SOX2, LIN28 and C-MYC were fused to the protein transduction domain of the HIV1-TAT. TAT recombinant proteins were produced in human embryonic kidney (HEK293) cells, to allow post-transcriptional modifications, and were purified with FPLC (Akta, GE Healthcare) using either a STREP or a 6-His tag. The purity and concentrations of TAT-proteins were assessed with Western blot and ELISA. Sox2 or Nanog promoter reporter lines were constructed with human fetal lung fibroblasts (IMR90, ATCC). Reporter IMR90 were cultured in fibroblast medium in 24-well plates at a density of 10.000 cells per well and were treated at 80% confluency with combinations of TAT-proteins and the following small molecules: CHIR99021 (3µM), PD0325901 (, BIX01294, SB31542 (25 µM), valproic acid (0.5 µM), sodium butyrate (1 mM), ascorbic acid (1 mM), 5-azacytidine and E-616452 (2.5-25 µM). Cells were treated daily for 5 days with the proteins and for 10 days with the molecules. At day 15, they were replated in hESC medium on mouse embryonic feeders (MEF). E-616452 at 2.5 µM was added after 5 days for 10 days. Controls received either: TAT-proteins only, small molecules only, or the culture medium only. Morphological changes were observed from day 5 and day 10 in wells treated with TAT-OKSL in the presence of CHIR99021+PD0325901+E-616452 and in the presence of VPA+SB431542+E-616452 (2.5 µM), respectively. Cell clusters formed from day 15 in both conditions and evolved into colony-like structures in 3 weeks. The efficacy of reprogramming was of 0.001% in both conditions. Adding TAT-c-Myc had no further effects. Other combinations resulted in either death of fibroblasts after plating in hESC conditions or formation of non-viable clusters of varying sizes. Strong fibroblasts death was observed in control wells with small molecules in the absence of TAT-proteins. The expression of pNANOG-GFP and pSOX2-GFP was concomitant to morphological changes, confirming rapid cell transduction and biological efficacy of the proteins, and was maintained for the whole duration of the experiments, suggesting accurate reprogramming. The analysis of the colonies is in progress, as is the validation of the process on other cell sources.

Poster Board Number: 3278

IDENTIFICATION OF A SET OF TRANSCRIPTION FACTORS FOR DIRECT REPROGRAMMING OF HUMAN SOMATIC CELLS INTO HEMATOPOIETIC STEM CELLS/PROGENITORS

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Modern methods of treatment of numerous malignant (e.g. leukemia and lymphoma), and genetic (e.g. immunodeficiencies, hemoglobinopathies, and Fanconi's Anemia) blood diseases rely on hematopoietic stem cell (HSC) transplantation. While it is possible to prospectively isolate HSCs/ progeni-

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tors from patient's blood and bone marrow to use for transplantations, they neither can be reliably maintained and expanded in undifferentiated state in culture nor manipulated to eliminate genetic cause of a disease. In order to overcome this problem we have attempted to rationally identify a set of transcription factors necessary for reprogramming human somatic cells into HSCs. During development the first definitive HSCs are found in the dorsal aorta within the aorta-gonad-mesonephros (AGM) region, in two arteries that connect the dorsal aorta with the yolk sac, and the vitelline and umbilical arteries. Mouse as well as human placentas and placental vasculature are considered to be potent hematopoietic sites from midgestation to birth. We have compared a transcription profile of human HSCs and human umbilical arterial endothelial cells (HUAECs). We have identified a set of transcription factors and micro RNAs that are differentially expressed in these two cell type. Over-expression of a subset of these factors in human embryonic fibroblasts converted them into CD45+ cells. In a methyl-cellulose colony forming assay CD45+ fibroblasts were able to generate CD11b+, CD16+, and CD71+ cells.

Poster Board Number: 3282

CHEMICAL AND GENETIC PLATFORMS FOR THE GENERATION OF PHARMACEUTICAL-GRADE, TRANSGENE-FREE HUMAN INDUCED PLURIPOTENT STEM CELLS

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The generation of induced pluripotent stem (iPS) cells by forced expression of reprogramming factors has overcome the ethical and technical hurdles that are associated with the use of embryonic stem cells. However, the use of iPS cells in scientific and clinical applications is hindered by the very low reprogramming efficiency, the use of viruses and oncogenic transcription factors to generate iPS cells, the incomplete silencing of the trangenes or their re-activation, and the use of animal products including serum and feeder cells. To overcome these obstacles and generate "pharmaceutical-grade" human iPS cells, we established an enhanced protocol for the generation, selection, and maintenance of iPS cells. Human fibroblasts were isolated and cultivated from donor-consented adult tissue (skin biopsy) or neonatal foreskin tissue, and infected with a single lentivirus carrying a Cre recombinase-excisable polycistronic cassette expressing three or four reprogramming factors (Oct4, Sox2, and Klf4, with or without c-Myc) connected with 2A self-cleaving peptides and internal ribosome entry site (IRES). The use of the polycistronic lentivirus reduced the number of viral integrations required for the reprogramming process, and thus reducing the risk of insertional mutagenesis. We achieved significant enhancement in reprogramming efficiency (more than 50 fold) by using a cocktail of 4 small molecule inhibitors of signaling pathways (TGFβ, MEK, GSK3β, and ROCK) that regulate stem cell differentiation and self-renewal. The small molecule cocktail enabled efficient generation of iPS cells in feeder-free culture and defined media, culture conditions that are required if iPS cells are to be used for pharmaceutical or clinical applications. In addition, instead of relying on morphology only for picking iPS cell colonies, we have improved on the selection process by using fluorescence-activated cell sorting for the isolation of SSEA4- and Tra-1-81 double positive iPS cells. To eliminate any residual expression of the transgenes or the risk of reactivation, established iPS cell-lines were transfected with the Cre recombinase to excise the polycistronic cassette (contains a loxP site) and transgene-free clones were selected and characterized. Thus, our chemical and genetic platforms enabled robust generation of feeder-free, transgene-free "pharmaceutical-grade" iPS cells that can be used in pharmaceutical screening applications, and ultimately cell therapy applications.

Poster Board Number: 3284

DIRECT REPROGRAMMING OF MOUSE FIBROBLASTS TO NEURAL PROGENITORS

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The simple yet powerful technique of induced pluripotency may eventually supply a wide range of differentiated cells for cell therapy and drug development. However, making the appropriate cells via induced pluripotent stem cells (iPSCs) requires reprogramming of somatic cells and subsequent re-differentiation. Given how arduous and lengthy this process can be, we sought to determine whether it might be possible to convert somatic cells into lineage-specific stem/progenitor cells of another germ layer in one step, bypassing the intermediate pluripotent stage. Here we show that transient induction of the four reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) can efficiently transdifferentiate fibroblasts into functional neural stem/progenitor cells (NPCs) with appropriate signalling inputs. Compared to "iN cells", transdifferentiated NPCs have the distinct advantage of being expandable *in vitro* and retaining the ability to give rise to multiple neuronal subtypes and glial cells. Our results provide a new paradigm for iPSCfactor-based reprogramming by demonstrating that it can be readily modified to serve as a platform for transdifferentiation.

Poster Board Number: 3286

APPROACH OF LINEAGE REPROGRAMMING OF MOUSE EMBRYONIC AND CARDIAC FIBROBLASTS INTO CARDIOMYOCYTES

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Introduction of iPSCs was beyond doubt a major breakthrough. Nevertheless direct conversion of one cell type into another without passing through the intermediate pluripotent stage is highly desirable for regenerative medicine. Recent success in direct lineage reprogramming in the pancreatic and neuronal field encouraged us to establish a project on direct lineage reprogramming into cardiomyocytes. We cloned 9 cardiac transcription factors (Nkx2.5, Gata4, Gata5, Tbx5, Tbx20, Mef2c, Myocd, Mesp1, Srf) and the chromatin remodeling factor Baf60c into lentiviral backbones. All possible 120 combinations of 3 factors out of this pool of factors were screened on E13.5 mouse embryonic fibroblasts (Mefs). As readout Q-PCR for the cardiac contractile apparatus genes Myh6, Myl2, Actc1 the transcription factor Nkx2.5 and the cardiac sodium channel gene Scn5a was applied. Upregulation of all 5 cardiac markers was considered as indication for a positive reprogramming event. The combination of Tbx5, Mef2c, Gata4 which was recently shown to result in beating cardiomyocytes by direct reprogramming of fibroblasts only caused upregulation of Myh6 and Actc1 whereas expression of Myl2, Nkx2.5 and Scn5a was unchanged in our experiments. In contrast we identified one alternative combination resulting in at least five times upregulation of all tested cardiac genes. Antibody staining against cardiac alpha-actinin was positive for both combinations but did not show organized striation. Also no spontaneous beating cells were observed. We reasoned that the starting cell type might be important for reprogramming into functional cells. Therefore a transgenic mouse model express-

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ing dtTomato under the control of the cardiac alpha-myosin heavy chain promoter (Myh6) was established. This enabled us to isolate and purify neonatal cardiac fibroblasts and furthermore to *in vitro* trace the induction of the cardiac program by the expression of dtTomato. As starting population cardiac fibroblasts were isolated from heart pieces and FACS sorted for Myh6 negative, Thy1 (CD90) positive cells. No dtTomato positive cells were detected in the cultures directly after sorting. Application of Tbx5, Mef2c, Gata4 resulted in 0.5% dtTomato positive cells 14 days post infection. With the combination identified in our screen we obtained 2% dtTomato positive cells. Antibody stainings against cardiac alpha-actinin 14 days post infection showed beginning of cross striation and therefore indicated the establishment of a contractile apparatus. Further analyses are necessary to figure out whether functional cardiomyocytes can be obtained with this method. Currently we are investigating the electrophysiological properties of the dtTomato positive cells.

Poster Board Number: 3288

A FULL MIRNA LIBRARY SCREEN REVEALS A NEW MIRNA FAMILY ENHANCING REPROGRAMMING OF MURINE CELLS

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Introduction: Induced pluripotent stem cells (iPSCs) can be generated by ectopic overexpression of the four transcription factors Oct4, Sox2, Klf4 and c-Myc. Even though re-activation of the pluripotency-related transcriptional network during reprogramming is well studied, only limited information is available on the role of microRNAs (miRNAs), which support induction of pluripotency in somatic cells. Here, conducting a full miRNA library screen during the early phase of iPSC generation, we investigated if particular miRNAs can improve reprogramming and sought to elucidate further molecular pathways involved in the induction of pluripotency. Materials/Methods: We transduced murine embryonic fibroblasts (MEFs) from OG2 mice (Oct4 promoter-driven GFP expression) with a polycistronic lentiviral construct expressing Oct4, Klf4 and Sox2 from a spleen focus forming virus (SFFV)-derived promoter/enhancer. Twenty-four hours after transduction, we individually transfected murine miRNAs from a Pre-miR miRNA Precursor Library-Mouse V3 containing 379 miRNAs (Ambion) into these MEFs. At day 7 to 10 after transduction, GFP-positive colonies were counted. Results: Applying a threshold of 4-fold induction of reprogramming events over the controls, we could identify miRNAs previously demonstrated to improve reprogramming, such as the mir-290 and mir-302 clusters. In addition, we were able to find twelve hitherto unpublished miRNAs profoundly enhancing iPSC generation. Interestingly, we identified a miRNA family targeting the same downstream mRNAs. Based on in-silico predictions (Targetscan and miRBase), we investigated the putative miRNA targets BMPRII, Mier1, Mecp2, Meox2, phospho-Erk1/2 and Pten by western blot and luciferase assays. Indeed, one of these factors was directly regulated by this miRNA family. Further validation reveals that ectopic overexpression of the protein from a lentiviral construct significantly decreased reprogramming events, but specific siRNA-mediated knockdown of this factor enhanced reprogramming efficiencies comparable to the miRNA-mediated effects. Conclusion: Applying a full library miRNA screen, we were able to identify further miRNAs that significantly increase the induction of pluripotency in MEFs. Indeed, a newly characterized miRNA family specifically targets one protein, whose knockdown facilitates and whose lentiviral overexpression blocked reprogramming. Further studies aim at elucidating downstream effectors of the transcription factor identified.

Poster Board Number: 3290

PROBING THE MICROENVIRONMENTAL CUES THAT INFLUENCE ACQUISITION OF PLURIPOTENCY IN ADULT MOUSE SPERMATOGONIAL STEM CELLS

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Adult spermatogonial stem cells (SSCs) maintain spermatogenesis in males and also transmit genetic information to the next generation. Despite their lineage commitment, these cells are capable of achieving pluripotency spontaneously, forming embryonic stem cell-like cells (referred to as multipotent adult spermatogonial-derived stem cells or MASCs), which in turn can produce a repertoire of tissues. Therefore, it is important to understand the genetic and/or environmental conditions that predispose these cells to acquire pluripotency without any prior manipulations. Identified by GPR125 expression, SSCs can either remain quiescent, self renew, undergo programmed cell death or form MASCs. Based on our established protocols to maintain SSCs *in vitro*, we hypothesize that the microenvironment created by the combination of SSCs and CD34+ mouse testis stromal cells, employed as feeders, determines the fate of SSCs. To address this hypothesis, we have utilized SSCs derived from transgenic reporter mice to monitor change in GFP expression, as an indicator of pluripotency gene promoter activity. Our study revealed a progressive increase in transcriptional activity of promoters associated with pluripotency in SSCs maintained in long term culture; this increase can be disrupted by passaging the cells and is highly dependent on initial plating conditions. These findings concur with prior observations that SSCs require uninterrupted maintenance in culture in order to acquire pluripotency. In order to understand the biology of the SSCs in normal culture conditions along with change in potency, we have investigated the change in cell cycle, cell proliferation and differentiation undergone by the SSCs. We have adopted three different approaches to define the niche-derived signals, as well as identify the molecular changes induced by these signals, that influence SSCs to form MASCs, a) altering culture conditions to influence cellular properties of SSCs, such as colony and monolayer formation, b) manipulating expression of candidate genes in SSCs and c) a chemical genetic approach. Our data suggest that the extracellular milieu strongly influences the SSC phenotype *in vitro*. Since the factors essential to support self-renewal of SSCs may be conserved among mammalian species, a deeper understanding of the biology of the SSCs could reveal alternative pathways to induce cells to become pluripotent, which would be of general importance in the field of stem cell biology and regenerative medicine

Poster Board Number: 3292

GENERATION OF A ZERO-FOOTPRINT AND XENO FREE INDUCED PLURIPOTENT STEM CELLS.

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The generation of induced pluripotent stem cells from fibroblasts or other somatic cells enables the possibility of providing unprecedented access to patient-specific iPS cells for drug screening, disease modeling, and cell therapy applications. Animal models of human disease have shown that these cells have a potential to treat conditions developed by ageing, birth defects and injury. However, one major obstacle to the use of iPS cells for therapeutic applications is the viral-based delivery of reprogramming factors that result in multiple proviral integrations that pose the danger of insertional mutagenesis. Second, the current reprogramming protocols involve the

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use of products of animal origin, making them unsuitable for the generation of clinical grade iPSCs. Here we report the generation of a zero-footprint iPSCs in a Xeno-free condition. We derived human foreskin fibroblasts under Xeno-free conditions, reprogrammed with non-integrating mRNA method, and expanded iPSCs under XF-human feeders and conditions. The iPSCs generated in this system are able to proliferate, maintain and displays pluripotent markers. Further these cells are able to give rise to EBs that can differentiate to all the three lineages - ectoderm, endoderm and mesoderm. We also demonstrate that these iPSCs are free of genomic integration of exogenous genes by ICC staining and PCR. Generation of a footprint free iPSCs under Xeno free conditions should facilitate the safe clinical translation of iPSC-based therapies

Poster Board Number: 3294

EXPERIMENTAL APPROACHES FOR THE GENERATION OF INDUCED PLURIPOTENT STEM CELLS WITH NON INTEGRATIVE VIRUS

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To date, a large number of reports have described reprogramming of many somatic cell types into induced pluripotent stem (iPS) cells, using different numbers of transcription factors and devising alternate methods of introducing the transcription factor genes or proteins into the somatic cells. However, one of the limitations of traditional reprogramming is that it leads to iPS cells that have integrated into their genome the viral genomes used for reprogramming, thus acquiring a mutagenic potential. Experiments leading to integration-free reprogramming of human somatic cells provided the proof of principle that transient expression of the four classical reprogramming factors is indeed sufficient to induce pluripotency in somatic cells. Nevertheless reprogramming efficiencies with current non integrating methods are several orders of magnitude lower (~0.001%) than those achieved with integrating vectors (0.1%-1%), most likely because factor expression is not maintained for a sufficient length of time to allow complete epigenetic remodelling. Here, we describe a method using mutated lentivirus which does not integrate into in the genome. The goal of our project is the validation of non-integrative vectors for iPS cell reprogramming. We are developping a new vectorology tool based on lentiviral transduction, but this time not integrative. Non-integrative lentiviral vectors (NILV) have been developed by several European teams and studies show they can be used as tools for effective transitive gene transfer. They retain their ability to penetrate effectively into the target cells while losing their capacity of integration into the host genome. In fact, mutations are introduced directly into the gene coding for the virus integrase. The transgene expression is obtained from episomal forms of viral genome that are gradually diluted during cell divisions. We are currently testing different ways to transduce somatic cells with the NILV and we use integrative virus as a control. For now, these tests are performed with a GFP reporter gene and we could determine an accurate" M.O.I (Multiplicity Of Infection) for transduction. This M.O.I leads to a level of intensity of non-integrated GFP expression similar to that observed with integrated GFP. For the reprogramming experiments we will use a polycistronic vector containing OCT4, SOX2, KFL4 and c-MYC in fibroblasts. If this method allows us to obtain iPS cells free of any genome injury these cells and their *in vitro* differentiation into defined cell lineages should have an even greater impact as model systems for human diseases. The long-term goal of iPS cell technology is to provide a source of stem cell-derived allogeneic or autologous cell grafts for regenerative medicine approaches.

Poster Board Number: 3296

REPROGRAMMING OF FIBROBLASTS BY NON-VIRAL TRANSFECTION OF MRNA ENCODING 4 REPROGRAMMING FACTORS

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Introduction: The groundbreaking work of Yamanaka's group (2006) proved the possibility to reprogram mouse embryonic fibroblasts into stem cell-like cells (induced pluripotent stem cells or iPS) by viral introduction of 4 types of plasmid DNA, each encoding a defined factor (Oct4, Sox2, Klf4, cMyc). Since then, laboratories all over the world tried to improve the efficiency or safety, among others by using other (e.g. Nanog, Lin28) and/or less factors, by using other transfection methods (e.g. non-integrating vectors, protein-based reprogramming). In the past decade, it became clear that mRNA, however always believed to be too unstable, can indeed transfect cells efficiently. Because mRNA does not integrate into the host genome, it is a perfect candidate to safely introduce the factors into somatic cells. Results: Our group has elaborate experience in mRNA transfection by non-viral means. Because mRNA induced protein expression is *in situ* transient, it is imperative that expression of the 4 factors lasts long enough to induce reprogramming. Therefore, we introduced mRNA encoding luciferase (by complexation with liposomes) in two cell lines (HeLa and MSCs) and ensured that luciferase levels were detectable up to 9 days post transfection. Then, we subjected fibroblasts to either a single, double or triple transfection with 4 different mRNAs, each encoding a factor (Oct4, Sox2, Klf4, cMyc). Flow cytometry following immunostaining proved successful transfection of all introduced factors, moreover, also expression of other transcription factors typically upregulated during the reprogramming process (such as Nanog) was significantly increased. Preliminary results, based on morphology and immunohistochemistry, indicate that iPS colonies have indeed been formed. Conclusion: mRNA not only has the potential to introduce the 4 above-mentioned factors in somatic cells and thereby induce their reprogramming, it also has several advantages, such as no risk of insertional mutagenesis and lower immunogenicity (no CpG motifs). Preliminary results are very promising with regard to iPS colony formation, the final proof by means of embroid body and teratoma formation is currently being performed.

Poster Board Number: 3298

COMPARATIVE INDUCTION OF PLURIPOTENCY IN FIBROBLASTS USING LENTIVIRUS AND BACMAM

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The majority of the methodologies used to reprogram somatic cells into Induced Pluripotent Stem Cells (IPSCs) rely on genetic modification via viral vector. There is a great interest to elucidate a highly efficient economical and bio-safe non-genetic modifying method in order to move the IPSC field from bench to bedside providing a therapeutic source of stem cells to treat human disease or injury. In this study, we compared the reprogramming of human fibroblastic cells using two different methods; lentiviral which modifies the genome, and a hybrid BacMAM system that leaves the cell unmodified. The latter system can be considered a potentially therapeutic mode to reprogram cells. We used the CMV promoter combined with the beta globin intron in both systems to drive the expression of four factors; Oct4, Sox2, KLF, and L-Myc. To help track viral transduction Oct4 was combined with FMDV 2A splicing domain fused to a novel RFP-Blasticidin S-Deaminase fusion protein

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(Oct4-2A-RB). In addition, we prepared a polycistronic counterpart combining Oct4, Sox2, KLF4 together with the 2A-RB domain, allowing visual tracking of transduction efficiency. Transduction of lentiviral four factors yielded greater than 80% infection rate, similar to that observed with BacMAM. As the polycistronic lentiviral vector was difficult to package, we were unable to efficiently infect the cells. However, BacMAM polycistronic virus was as efficient as the four factor mix at transduction. Following transduction and 5-7 day treatment with VPA, the cells were passaged from a serum containing fibroblast media into mTeSR1/Matrigel with 3 small molecules to enhance reprogramming. After 7 days, the majority of the BacMAM infected cells were either dead, or no longer fluorescent, whereas the four factor lentiviral infected cells showed early signs of colony formation. After 30 days, we observed distinct colonies, which were picked and expanded in mTeSR1 on Matirgel coated plastic. Current work is aimed at characterizing the new IPSC line, and solving the massive cell death observed after BacMAM infection by addressing the role of interferon pathways activated via innate immune response to viral infection.

Poster Board Number: 3300

DERIVATION OF DOPAMINE NEURONS USING DEFINED FACTORS

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Parkinson's disease (PD) is a neurodegenerative disease affecting predominantly older people and is characterized by loss of movement control. The symptoms are caused by the degeneration of midbrain dopaminergic neurons of the substantia nigra pars compacta which are involved in the control of voluntary movement. Studies have shown that dopamine neurons can be derived from ES cells and iPS cells and successfully integrated into animal models of PD to improve Parkinsonian behavior. Previously, our lab has shown the direct conversion of fibroblasts to neurons with 3 transcription factors Brn2, Ascl1 and Myt1L. Here, we aim to generate DA neurons of the A9 group from mouse embryonic fibroblasts (MEFs) using defined factors. By screening known transcription factors implicated in determination of dopaminergic fate, we have identified factors that not only convert MEFs to cells with neuronal characteristics, but with unique dopaminergic markers as well. Electrophysiological studies and functional transplantation into animal models will be used to characterize these cells. Our results suggest a promising future for these cells in cell replacement therapy of Parkinson's disease. This strategy can also be used to generate specific subtypes of neurons that are involved in other diseases.

Poster Board Number: 3302

POU5F1 EXPRESSION IN PORCINE FIBROBLASTS ACCELERATED NUCLEAR REPROGRAMMING IN PORCINE NUCLEAR TRANSFER DERIVED EMBRYOS

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Efficiency of reproductive cloning derived from nuclear transfer using undifferentiated status cells, i.e. embryonic stem cells has been known higher than differentiated ones in mice. It is believed that early embryonic genes (Oct4, Sox2 and Nanog, etc) may have beneficial influence on its nucleus reprogramming to produce cloned offspring. In this study, we hypothesized that ectopic expression of an early embryonic gene, oct4 in neonate skin fibroblasts will have affect early nuclear transfer embryonic development in pig. For this study, coding domain region of porcine oct4 from pig ovarian cDNA by PCR was cloned, sequenced and ligated to pCMV-IRES-DsRED express II vector (Clontech, USA). The linearized DNA was transfected to neonate ear skin fibroblasts and then after 48 hours 1mg/mL, neomycin was added in culture medium for around two weeks. Oct4 expression was observed in nucleus after immune-staining in selected fibroblasts, changed the morphology of the cells, and increased cell proliferation without viability decrease. Expression of related other genes (Sox2, c-Myc and Nanog) was not changed. Nuclear transfer was carried out using transfected (oct4 expressed) or non-transfected cells as followed to our previous established protocol. Cleavage rate of cloned embryos from oct4 expressing cell line was higher than control group. Interestingly, first blastocyst formation time is one day faster than control group. In conclusion, this study demonstrated that porcine oct4, which was cloned and sequenced for the first time, had not only affected the morphology and proliferation of neonate skin fibroblasts, but also accelerated nuclear reprogramming to produce nuclear transfer embryos. In the future, we will find out which mechanism may be connected in increase of speed of nuclear transfer embryonic development. This study was supported by grants from MKE (#2009-67-10033839, #2009-67-10033805), IPET (#109023-05-1-CG000), NRF (#M10625030005-10N250300510), and BK21 program.

Poster Board Number: 3304

DERIVATION AND CHARACTERIZATION OF PARTHENOGENETIC EMBRYONIC STEM CELLS FROM RHESUS MONKEY

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The present study showed the rhesus monkey (Macaca mulatta) parthenogenetic embryonic stem cells (rpESCs) lines were derived from parthenogenetic blastocysts in serum-free medium. These two novel rpESCs have typical primates ESCs morphology including clear clones boundary, high nuclear-cytoplasm ratio and significant nucleolus. The rpESCs are positive for alkaline phosphatase and are immunoreactive for octamer-binding transcription factor 4 (Oct-4), Nanog, tumor rejection antigen 1-60 (TRA 1-60), tumor rejection antigen 1-81 (TRA 1-81), stage-specific embryonic antigen 4 (SSEA-4); The cells also were detected Sox-2 and Rex-1 by RT-PCR. The two novel rpESCs could spontaneously differentiate into cell lineages representative of all three embryonic germ layers by embryonic body (EB) formation and teratoma. Detailed G-banding analysis revealed that all of the two ESC lines were karyotypically normal, with a diploid set of 40+XX chromosomes, even after long time cultivation *in vitro* and repeat freezing. Several imprinted genes were tested and results suggested that both of the rpESCs expressed maternally imprinted genes including H19 and UBE3A. However, paternally expressed imprinted genes have a variable expression pattern, such as both of the rpESCs expressed PEG3 and SGCE, whereas, PEG10, ZIM2, and MEST were detectable only in one of the rpESCs. In addition, SNPRN, NDN and MAGEL2 were absent in both of rpESC but expressed in control biparental ESCs. These results suggest that reactivation of paternally imprinted genes in parthenogenesis was a stochastic process.

Poster Board Number: 3306

EXPRESSION AND METHYLATION PATTERN OF IMPRINTED GENES DURING *IN VITRO* DIFFERENTIATION OF PARTHENOGENETIC INDUCED PLURIPOTENT STEM CELLS

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Induced pluripotent stem (iPS) cells are generated from somatic cells by introducing a small number of transcription factors. Many iPS cell clones

have been generated using different tissues. We previously generated iPS cells from bimaternal parthenogenetic neural stem cells (pNSCs). Here we show the epigenetic status of imprinted genes influenced their gene expression during the generation and differentiation of parthenogenetic iPS cells. As expected, there was no the difference between NSC and pNSC on the expression of paternal imprinting genes. However we found that maternal imprinting genes exhibited remarkably higher expression in NSC, whereas they were expressed in pNSC at lower levels than in NSC through heat map of imprinting genes. In addition, parthenogenetic iPS cells exhibited a global gene profile clustering and an imprinting gene profile clustering closer to pESCs clustering and that was clearly distinct from that of pNSC. Interestingly, pNSCs lost the parthenogenetic imprinting patterns during pluripotential reprogramming. Parthenogenetic iPS cells had not reverted to the parthenogenetic imprinting patterns during differentiation. We explored the correlation between expression and methylation of PWS-related imprinted genes during reprogramming and differentiation. These results indicate that parthenogenetic iPS cells may be a useful tool to study Prader-Willy syndrome and other genetic imprinting diseases.

Poster Board Number: 3308

DIRECT LINEAGE CONVERSION OF HEPATOCYTES TO FUNCTIONAL NEURONS

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We recently show that mouse fibroblasts can be directly reprogrammed into induced neuronal (iN) cells by the three transcription factors Ascl1, Brn2 (Pou3f2), and Myt1l. This suggests that direct cell fate conversions are possible even between distantly related somatic cell types. However, this study left the important question open, what the differentiation stage and exact nature of the donor cells were. Primary fibroblast cultures are a heterogeneous mixture of various, presumably mesenchymal progenitors potentially including neural crest-derived lineages. We therefore asked whether iN cells can be generated from a better defined, terminally differentiated cell type. Here, we show that the same transcription factors can also convert albumin-expressing hepatocytes into neuronal cells (Hep-iN cells). This provides unequivocal proof for direct lineage conversion between somatic cell types representing different germ layers.

Poster Board Number: 3310

MOLECULAR MECHANISMS OF ASTROGLIA-TO-NEURON CONVERSION BY FORCED EXPRESSION OF NEUROG2

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Direct conversion of endogenous glia into neurons could provide an alternative for cell-based therapies of neurodegenerative diseases. One glial population meeting the requirement of widespread distribution throughout the mammalian brain consists of astroglial cells. Astroglial cells may be particularly suitable for attempts of direct conversion into neurons as they are lineage-related to neurons through common radial glia ancestors, they share molecular properties with astro/radial glial stem cells in the developing and adult brain and can acquire hallmarks of stem cells following injury and isolation *in vitro*. We have recently shown that retrovirus-mediated expression of the proneural genes Neurog2 or Mash1 (also referred to as achaete-scute complex homolog 1[Ascl1]) can directly convert astroglia of the postnatal mouse cerebral cortex into fully functional neurons. In order to gain insight into the molecular mechanisms of proneural gene-mediated direct astroglia-to-neuron conversion, we took advantage of an inducible system in which the Neurog2 coding sequence is fused to a modified estrogen receptor (ERT2)-binding domain (Neurog2ERT2), rendering Neurog2 activity dependent on tamoxifen treatment. We could show that this system is tight in that Neurog2 activity can only be observed following stimulation with tamoxifen. Moreover, several days of tamoxifen treatment is capable of inducing neurogenesis from astroglia to a similar extent as forced expression of the wild type Neurog2. Using this system, which allows for a more precise timing of Neurog2 activity, we are currently examining which genes are activated during the course of direct astroglia-to-neuron conversion. Using this inducible system we also observed that, in contrast to an immediate onset of Neurog2 activity, delayed induction of Neurog2ERT2 by tamoxifen failed to convert postnatal astroglia into neurons. This loss of susceptibility to Neurog2-induced reprogramming may be due to progressive astroglial maturation, as reflected by a massive up-regulation of GFAP expression, providing a model system to study the dependence of proneural gene-mediated reprogramming on the maturation state. Morerover it allows for examining the possibility to overcome the loss of plasticity by forced expression of additional transcription factors.

Poster Board Number: 3312

INHIBITION AND ENHANCEMENT OF REPROGRAMMING BY CHROMATIN MODIFYING ENZYMES

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Generation of induced pluripotent stem (iPS) cells from differentiated cell types by somatic cell reprogramming involves complete resetting of epigenetic states. A number of proteins are known to play a role in regulating the chromatin marks associated with these distinct epigenetic changes, but how such epigenetic modifiers influence the reprogramming process remains largely unknown. To address this question, we generated a shRNA mini-library targeting genes in DNA and histone methylation pathways, and screened for positive and negative regulators of iPS cell generation. Knock-down of the H3K27 histone methyl-transferase Ezh2 and partner components of the polycomb repressive complex inhibited reprogramming, while knockdown of three genes markedly increased reprogramming efficiency. Reduced expression of one such gene, the H3K79 histone methyltransferase Dot1L, not only significantly increased the yield of iPS colonies, but could also substitute for the reprogramming factors Klf4 and Myc. iPS cells generated with only Oct4 and Sox2 in the presence of Dot1L inhibition differentiated into all 3 three germ layers in teratoma analyses, indicative of their pluripotency. We have established that Dot1L inhibition functions early in the reprogramming process, when gene expression analyses reveals a marked induction of the two alternative reprogramming factors Nanog and Lin28. By loss-of-function experiments, we showed that Nanog and Lin28 play essential functional roles in the enhancement of reprogramming by Dot1L inhibition. Our findings implicate select epigenetic modifiers in somatic cell reprogramming. Modulation of these genes can be exploited to generate iPS cells more efficiently and with fewer exogenous transcription factors, and to understand the epigenetic mechanisms at play during reprogramming.

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Poster Board Number: 3314

REPROGRAMMING OF LINEAGE MARKED DIFFERENTIATED MUSCLE TO THEIR PROGENITOR CELLS BY SMALL MOLECULE INHIBITORS

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Reprogramming of terminally differentiated cells to their progenitors can serve as powerful source of tissue specific stem cells. Skeletal muscle is a classic example of differentiation which involves activation and proliferation of adult muscle stem cells that give rise to precursor cells (myoblasts) which exit cell cycle and fuse to form terminally differentiated multinucleated myotubes. In order to study muscle reprogramming in culture conditions, we developed a novel technique to preferentially label myotubes by using Cre-Lox method which unequivocally distinguished mononucleated cells (reserve cells) from multinucleated myotubes. A significant percentage of these lineage marked multinucleated myotubes then de-differentiated into proliferating myoblasts in the presence of a tyrosine phosphatase and a general caspase inhibitor. The reprogrammed mononucleated proliferating cells down-regulated muscle differentiation markers, such as myogenin and embryonic myosin heavy chain, decreased expression of cdk inhibitor p21 and up-regulated muscle progenitor markers such as Pax7 and MyoD1. These reprogrammed cells retain their full competence to form de-novo myotubes and contribute to *in vivo* muscle regeneration in immuno-deficient NOD- SCID mice. This de-differentiation of lineage marked myotubes into reprogrammed myoblasts by small molecules has broad implications in understanding adult tissue regeneration and can be translated towards novel therapeutic approaches aimed to enhance muscle repair.

EMBRYONIC STEM CELLS CLINICAL APPLICATION

Poster Board Number: 3316

SCALABLE CULTURING AND DOWNSTREAM PROCESSING FOR THE PRODUCTION OF HUMAN EMBRYONIC STEM CELLS

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As demonstrated by the FDA approval of two clinical trials, human embryonic stem cell (hESC)-based cell therapies are progressing from bench to clinic. However, current plate and T-flask-based culture platforms and open centrifugation processes severely limit the scalability of hESCs production to commercially relevant lot sizes at reasonable cost of goods. The Lonza cell therapy R&D team set out to develop a closed and scalable hESC manufacturing process. This process design includes culturing hESCs in multilayer cell factories, the culture platform widely used in current cell therapy manufacturing, automatically washing and concentrating cell harvest using scalable downstream processing technology. Traditionally, hESCs are harvested and passaged as colony fragments with enzymatic treatment followed by labor intensive mechanical scraping. Single-cell passaging and harvesting is not desirable due to the low post-passaging and cryopreservation recovery and the increased risk of abnormal karyotype associated with low cloning efficiency of hESCs. However, mechanical scraping cannot be directly applied in closed system and multilayer cell factories. To overcome this hurdle, we have optimized a process of expanding and passaging hESCs from T-flasks into multilayer cell factories with a novel non-enzymatic cell passaging method, followed by downstream processing with continuous counter-flow centrifugation technology. The post-detachment cell viability was 93.0% with our non-enzymatic passaging method, compared with 52.7% cell viability of hESC colony fragments generated with the conventional hESC enzymatic and scraping passaging method. This greater viability results in improved post-passaging attachment and recovery, which enables higher passage ratio, and thus leads to greater process efficiencies and a more rapid scale-up to harvests of billions of healthy, viable cells. The hESCs harvested from multilayer cell factories were characterized with flow cytometry and over 90% of these cells were expressing pluripotency markers including OCT4, SSEA4, Tra-1-60 and Tra-1-81. The final cell harvest was concentrated over 14 times after automated downstream processing, with only 2% drop in cell viability. Our study shows that hESC culture can be brought into scalable manufacturing platforms while maintaining the important quality parameters of viability and pluripotency markers. The culturing and downstream processing system we have developed has a great potential to be translated into cGMP compliant hESC production.

Poster Board Number: 3318

METHOD OF IMPROVING VIABILITY AND PURITY OF HESC-DERIVED NEURAL CELLS FOR CLINICAL TRANSPLANTATION

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The viability and purity of a human embryonic stem cell (hESC)-derived therapeutic product intended for clinical transplantation are important safety considerations. Neural cultures typically undergo some form of dissociation when being prepared for clinical transplantation. Neurons cultured in-vitro are especially sensitive to enzymatic dissociation and mechanical manipulations. Following such disruptions, immediate viability is 75% to 90%. Additional cell death occurs a few hours following dissociation, resulting a drop in viability to 60 to 80%. Therefore, it can be anticipated that transplantation of the dissociated population will include 10% to 40% nonviable cells, which is undesirable. In addition, hESC-derived neural populations for clinical use have the potential risk of containing unintended undifferentiated stem cells, which can present tumorigenic risk. Methods to purify hESC neural derivates, including FACS or other labeling methods, can introduce undesirable components in the transplant or more often results in a drop of viability. Here we present an inherent aggregation-based method that ensures the purification of neural cells by elimination of nonviable cells and viable cells outside of the neural lineage, including that of undifferentiated stem cells, without the use of labeling techniques. Following this method of purification and improved viability, the final transplant population has a viability of 99% to100%. This method provides advantages over other currently used methods for purification and improvement of viability of hESC-derived neural cell populations in development for clinical application.

Poster Board Number: 3320

DERIVATION OF A HUMAN EMBRYONIC STEM CELL LINE IN XENO-FREE CULTURE SYSTEM

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INTRODUCTION: Derivation and culture of human embryonic stem cells (hESCs) without animal material would be optimal for future application of hESCs in clinical cell therapy. Here, we set out to develop a protocol to

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derivation and maintain human embryonic stem cells that would be xeno-free condition. METHODS: We used post-natal, commercially available human foreskin fibroblasts (HFFs) as feeder cells. Thawed human embryos which survived and developed up to the blastocyst stage were cultured on a confluent layer of mitomycin C inactivated HFFs in G-1/G-2 embryo culture media in 4-well plates. On day 6, embryos developed into blastocysts with a cavity, zona pellucida was removed after a short incubation in acid Tyrode's solution. We developed a practical mechanical isolation method for the inner cell mass (ICM). Two glass pipettes with sharpened tips were used to device trophectoderm and ICM under a stereomicroscope. Isolated ICMs were cultured in xeno-free culture systems that replaced other reagents of animal origin (gelatin, Knockout SR, mouse fibroblast) with their recombinant equivalents (humanized CELLstartTM, Knockout SR xeno-free, HFFs). RESULTS: Cryopreserved human embryos (n=40) were donated to this study upon informed consent by couples. From these embryos, 9 blastocysts (2 early blastocysts, 2 mid blastocysts and 5 expanded blastocysts) were used for derivation of new clinical-grade hESC line. Three ICM outgrowths were mechanically passaged, and one hESC lines was derived (mid blastocyst). One xeno-free hESC line, which could be continuously passage in xeno-free conditions and maintained characteristics indistinguishable from previously established hESCs, including colony morphology and growth behavior, expression of pluripotency-associated markers, normal karyotype (46,XY) and pluripotent differentiation ability *in vitro*. CONCLUSION: Overall, the results presented here demonstrate that hESCs can be generated and maintained under xeno-free conditions that should be facilitated the clinical translation of hESCs-based therapies. This work was supported by the Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology, Republic of Korea (SC1150).

Poster Board Number: 3322

HIGH QUALITY EPISOMAL HUMAN IPSC LINES DERIVED FROM CORD BLOOD WITH A ZERO VIRAL FOOTPRINT: APPLICATIONS IN SERUM FREE AND XENO FREE CULTURE SYSTEMS

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Nonviral, non-integrated human induced pluripotent stem cell (hiPSC) lines were generated using cord blood-derived CD34+ progenitors with seven episomal expressed factors (OCT4, SOX2, KLF4, MYC, NANOG, LIN28, SV40 T) expressed with the pCEP4 plasmid system. Zero foot print iPSC lines were derived first on feeders and subsequently adapted to feeder free conditions in serum free and xeno-free culture conditions. In all three culture conditions, the iPSC lines showed robust proliferation with normal karyotype and expressed a panel of pluripotency markers Oct4, Sox2 and Nanog by RT-PCR and Oct4, SSEA4, TRA-1-60 and TRA-1-81 by ICC. Whole genome expression and epigenetic profiling analyses demonstrated that these lines were of extremely high quality and molecularly indistinguishable from human embryonic stem cell lines. In a directed differentiation and teratoma analysis, the lines retained potential to the three germ layers, ectoderm, endoderm and mesodermal lineages. This report presents an optimized culture system for nonintegrated human iPSC in a feeder -free, serum - free and Xeno-free conditions, that enable the use of iPSC line as a positive control in reprogramming methods for iPSC generation. In addition, viral free vascular, hematopoietic, neural and cardiac lineages were derived with robust efficiencies that support cell therapy applications.

Poster Withdrawn

Poster Board Number: 3328

COMPARATIVE PROTEOMICS OF KARYOTYPICALLY ABNORMAL AND NORMAL HUMAN EMBRYONIC STEM CELLS

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Previous reports indicated that during long term culture, the human embryonic stem cells (hESCs) acquired genomic alterations and the transformation hESCs had a tendency to progression to malignant cells, which raise concerns about the safety of future clinical use of the cultured hESCs, but which may be an excellent model for characterizing the initial stages that determine the transition of embryonic stem cells into cancer stem cells. In this study, ITRAQ-based tandem mass spectrometry was employed to quantify proteins of normal and aberrant karyotypic hESCs from simple to complex karyotype abnormalities. Over 2600 proteins were identified and quantitated, among which are proteins associated with pluripotency such as LIN28 and THY1 and development as well as FGF signaling and EGF receptor pathway. The expression levels of 313 proteins representing at least 24 molecular function groups including chaperone, kinases, proteases, ligase, calcium binding, nucleic acid binding and cytoskeletal proteins were found to be significantly different between normal and abnormal hESCs. Examples of some early developmental markers high in normal karyotypic hESCs include

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NES, GRB2, RBM14 and HNRPA3. Several proteins related to hyperproliferation and suppression of apoptosis such as HDAC2 were highly expressed in abnormal hESCs, but genes associated with apoptosis such as HNRNPK, DFFA and TKT were down-regulated in the karyotypically abnormal hESCs. In addition, several proteins that were highly expressed in abnormal hESCs such as DNMT3B, RAP2B, CTNNB1, CSNK2A1, PRDX1, EEF1A1, VDAC2, ANXA2 and YWHAZ have also been attributed to malignancy in other systems. Furthermore, 15 differential proteins were verified by real-time PCR and Western blot analysis was used to validate the proteomic analyses for a subset of the proteins including DNMT3B, NES, CTNNB1, EIF3B and DNMT1. In summary, this is the first large-scale quantitative proteomic analysis of normal and aberrant karyotypic hESCs, and the information generated by this study should serve as a useful reference that malignant transformation process of aberrant karyotypic hESCs has close relationship with the changes of DNA methylation, cell cycle and apoptosis, WNT, P53 and FAS signaling pathways, which may mirror those of oncogenesis and tumor progression. Also, dysregulation of DNMT3B, NES, and CTNNB1 could be detected as early as the pre-neoplastic stage.

Poster Board Number: 3330

HUMAN AMNIOTIC FLUID CELLS SUPPORT EXPANSION OF HUMAN EMBRYONIC STEM CELLS IN XENO-FREE CULTURE CONDITIONS

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Human embryonic stem cells (hESCs) have been routinely cultured on mouse embryonic fibroblast (MEF or STO) feeder layers with a medium containing animal products. However, for clinical application of hESCs, animal-derived materials from the animal feeder cells, animal substrates such as gelatin or Matrigel and medium containing animal serum are essential to be eliminated in the current culture system. In this study, we performed that hES cell lines, SNUhES4, SNUhES31 and H1 were cultured on human amniotic fluid cells (hAFCs) derived feeder layer with Knockout SR XenoFree as serum replacement and a humanized CELLstart as cell substrate. All of hESCs were able to propagate up to 10 passages on hAFCs feeders with xeno-free conditions. These hESCs were conserved normal karyotype and were expressed pluripotent stem cell markers, alkaline phosphatase (ALP), SSEA-4, TRA-1-60, TRA-1-81, Oct-4, and Nanog like hESCs cultured on STO or human foreskin fibroblast (HFF) feeders. In addition, we observed the expression of nonhuman N-glycolylneuraminic acid (Neu5GC) by flow cytometery, which was xenoantigenic components of contamination in hESCs cultures, was not detected in xeno-free condition. In conclusion, we demonstrated that hESCs could be successfully prolonged on hAFCs and excluded animal components for humanized culture conditions. It will be aided in the progression toward clinical-grade hESCs culture. This research was supported by grants (SC1150) from the Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology.

Poster Board Number: 3332

MAINTENANCE AND PROPAGATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS USING STEMADHERE XF™, A DEFINED AND XENOBIOTIC-FREE HUMAN RECOMBINANT PROTEIN

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In order to establish clinically adaptable methods for therapeutic applications of stem cells, there is increasing interest in completely defined and xenobiotic-free methods for stem cell expansion and differentiation. Nagaoka et al. have previously shown that E-Cadherin fused to mouse IgG Fc domain is an excellent substrate for maintenance and expansion of human embryonic (hES) and induced pluripotent (hiPS) stem cells. Extending this work, Primorigen has developed StemAdhere XF™, a defined and xenobiotic-free substrate useful for routine maintenance and expansion of hES and hiPS cells. StemAdhere XF™ can be handled at room temperature, is compatible with gentle, non-enzymatic passaging methods and works well with many defined and xenobiotic-free stem cell media. Here we present comparative data demonstrating StemAdhere XF™ as a robust substrate for maintenance and expansion of pluripotent stem cells in defined and xenobiotic-free culturing conditions.

Poster Board Number: 3334

ECTOPIC PREGNANCY CONCEPTUS AS A NOVEL SOURCE OF FETAL STEM CELL RESEARCH AND THERAPY

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Introduction: Regenerative medicine as a background of stem cell research and therapy, has a long history. Several diseases including Parkinson's disease, heart diseases, multiple sclerosis, spinal cord injury, diabetes mellitus and etc; are candidates to treat by different types of stem cell. Several sources of stem cell have been described such as bone marrow, umbilical cord, peripheral blood, germ cells and the embryo / fetus. Despite the potential benefit of human embryonic / fetal stem cells in cell therapy, the utilization of this source of stem cell remains controversial because abortion and termination of pregnancy under a normal condition is forbidden by nearly all the major world religions such as Islam and laws in most of Muslim countries permit termination of pregnancy and abortion only when the life of the mother is severely threatened or when continuing pregnancy may lead to the birth of a mentally retarded, genetically or anatomically malformed child. According to the mentioned rules and conditions in Islamic countries, finding a viable source for embryonic / fetal stem cell will be too difficult. On the one had Muslim scientists have an ideal as the finding a genetically and anatomically normal embryo / fetus as a source for stem cell research and therapy, but on the other hand, they should adhere to the law and related rules in all parts of stem cell research and therapy. Our purpose is that Ectopic Pregnancy (EP) conceptus can meet these major priorities. Ectopic Pregnancy: The incidence of Ectopic pregnancy (EP) is around 2% in all pregnancies. EP occurs when the conceptus implants and matures outside the endometrial cavity that is the cause of 10% of all pregnancy-related deaths because of the potential for tubal rupture and serious maternal mortality and morbidity.

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So EP has to be terminated because of maternal life saving. There are several well-known factors that are associated with risk of ectopic pregnancy such as Pelvic inflammatory disease (PID), a history of previous EP, tubal ligation, tubal surgery, intrauterine devices, ovulation induction, *in vitro* fertilization (IVF), Smoking and maternal age (more than 35 year - old). Our hypothesis is that when an ectopic conceptus has a chromosomal abnormality that is lethal in origin, the EP will regress spontaneously without the necessity of surgical intervention. As we described, EP is a valuable source because there are no cytogenetic or other etiologies that could affect the material and results of research and therapy. Conclusion: The authors suggest that using of EP material as a source of stem cell research and therapy has some advantages. Termination of an ectopic pregnancy is accepted by nearly all of Islamic laws because of maternal life saving, and also EP conceptus is a novel and valuable source for stem cell research and therapy.

Poster Board Number: 3336

BANKING CLINICAL GRADE HESC LINES: GOVERNANCE, REGULATORY AND ETHICAL ISSUES

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Stem cell research is steadily entering clinical translation with the launching of the first clinical trial (Phase I) testing a hESC- derived cell therapy by Geron and a second trial by Advanced Cell Technology Inc's recently approved by the FDA. Stem cell banks are increasingly seen as an essential resource of biological materials for both basic and translational research. They also support international access to authenticated, quality-controlled and ethically sourced stem cell lines. In short, clinical grade hESC banks are vital to the advancement of translational efforts. While regulatory frameworks for stem cell translation are being refined, regulatory uncertainty could create barriers to not only the availability of lines and their commercialization but also to the clinical development of cell therapies, thereby stressing the need for clear and harmonized definitions of key concepts and terms. Stem cell banks can only be effective if the lines they curate have undergone rigorous screening, derivation and manufacturing processes together with robust ethical provenance. Given the aforesaid consideration, the International Stem Cell Banking Initiative sponsored by the International Stem Cell Forum, is currently elaborating global best practices for the development of banks for clinical grade stem cell lines. The overall objectives are to enable the global sharing of hESC lines of high scientific quality and to foster compliance with regulatory and ethical requirements across heterogeneous legal and ethical frameworks. This presentation will focus on the results of the International Stem Cell Banking Inititiative's (ISCBI) survey of the policy, regulatory and governance frameworks related to clinical grade hESC in 16 jurisdictions. To that end, convergence and divergence in issues surrounding determination of compliance (i.e., provenance, access, ethics review, oversight), informed consent (i.e., withdrawal and re-consent requirements) as well as mechanisms for the sharing of samples and data will be analyzed. Finally, we will report on the governance and policy recommendations adopted by the ISCBI in its "Points to Consider in the Development of Banks for Clinical Grade hESC lines").

Poster Board Number: 3338

EXPRESSION OF EMBRYONIC STEM CELLS MARKERS IN INFANTILE HEMANGIOMA

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Background: Infantile hemangioma (IH), the most common tumor of infancy, is a tumor of the microvasculature. It typically undergoes rapid proliferation during infancy, followed by spontaneous involution. We have shown proliferating IH consists of a primitive mesoderm-derived hemogenic endothelium, with the presence of mesenchymal and hematopoietic stem cells. Primitive mesoderm-derived hemogenic endothelium is derived developmentally from embryonic stem cells (ESCs) during early fetal development. Aims & Objectives: To investigate the expression of ESC-associated proteins in proliferating IH, and to determine their ability to form teratomas *in vivo*. Methods: Proliferating IH biopsies from 6 patients were (i) processed for IHC staining for ESC-associated proteins Oct-4, SSEA-4, Nanog and STAT-3; (ii) used to determine the relative expression of Oct-4 & Nanog transcripts by qRT-PCR; and (iii) grown as explants prior to being injected into SCID/NOD mice. Results & Observations: IHC showed that the endothelial layer of proliferating IH expressed Oct-4, SSEA-4 and STAT-3. Cells within the interstitium, away from the endothelium, expressed Nanog. qRT-PCR confirmed the expression of mRNA transcripts for both Oct-4 and Nanog. All injected SCID/NOD mice formed tumors, but no teratomas were seen. Conclusion & Discussion: The mRNA and protein expression of ESC-associated markers in proliferating IH supports the developmental anomaly origin of IH. The expression of OCT-4, SSEA-4 and STAT-3 on the capillary endothelium and the expression of more downstream ESC marker, Nanog, on the interstitial cells, infer an ESC-like origin of IH. The novel demonstration of ESC markers on 2 distinct populations in proliferating IH and their inability to form teratomas *in vivo* suggests that IH are possibly derived from cells downstream of ESC phenotypes. The 10% prevalence of IH in the Caucasian population provides a novel extra-utero phenotype downstream of ESC for studying human developmental and regenerative medicine.

EMBRYONIC STEM CELL DIFFERENTIATION

Poster Board Number: 3342

IDENTIFICATION OF MICRORNAS REGULATING DIFFERENTIATION OF HUMAN NEURAL STEM CELLS

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MicroRNAs (miRNAs) are a class of small non-coding RNAs that act as post-transcriptional regulators of gene expression. Studies in model organisms have demonstrated critical roles for a number of microRNAs in neuronal development; however data on microRNA functions in the context of human neural development and neural stem cell biology are still scarce. We assessed microRNA signatures of human ES cells (hESC, I3 line), of derived long-term self-renewing neural epithelial stem cells (It-NES®) and of further differentiated neuronal cultures (ND15, ND30) using a quantitative real-time PCR multiplex assay. We validated the identified expression patterns of several microRNAs by Northern blot analysis in two independent hES cell lines (I3

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and H9.2) indicating the reliability of our approach. Furthermore, we were able to confirm previous assignments of ES cell-specific and brain-specific microRNAs. In addition to the known neurogenesis-associated microRNAs (i.e. miR-124 and miR-125), we identified novel microRNAs as up-regulated during human neural stem cell differentiation. Stable over-expression of a many of these newly identified microRNAs, just like the over-expression of miR-124 and miR-125, resulted in an increased rate of neuronal differentiation. This work shows that our human embryonic stem cell based neural differentiation protocol provides a useful paradigm for the analysis of microRNA profiles in human pluripotent and multipotent stem cells and for the identification and functional characterization of novel microRNAs associated with neuronal differentiation.

Poster Board Number: 3344

DISSECTION OF THE EARLY CARDIAC DEVELOPMENTAL PROCESS FROM HUMAN EMBRYONIC STEM CELLS USING A MODEL SYSTEM THAT RECAPITULATES THE EMBRYOGENESIS

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Human embryonic stem cells (hESCs) are expected to be a valuable model to study the embryonic development including cardiogenesis in culture. This is because the hESCs potentially have ability to differentiate into specific tissue cells along the *in vivo* embryonic developmental process, when cultured under the condition mimicking the embryonic environment. We have recently shown that cardiomyocytes developed from anterior primitive streak (PS) populations induced by synergistic activation of β-catenin using a glycogen synthase kinase (GSK) 3 inhibitor and blockage of bone morphogenetic protein (BMP) signaling by Noggin in hESCs. Now, we are mainly focusing on the following two topics. First, identifying the origin of cardiomyocytes is one of the important steps to understand the cardiac developmental process, but the lack of specific and suitable cell surface markers hampers identifying the origin of the heart-forming cells. To address the issue, we investigated the expression of cell surface proteins at different stages of cardiac development by flow cytometry analysis. We found that some markers, such as KDR, PDGFRα, PDGFRβ, CD56, CXCR4, and Integrinα6, may be useful to define the anterior PS populations that include the cells having cardiac potential. Second, recently, many researches reveal the existence of cardiovascular progenitors that can undergo bipotential or tripotential differentiation toward cardiomyocyte, smooth muscle, or endothelial lineages *in vitro* and *in vivo*. To examine the multipotentiality of the cells induced by our method, we established a single cell lineage tracing system that enables monitoring the differentiation from a single cell by the observation of the continued expression of GFP protein. Using this system, we found that one GFP-marked cell isolated from the anterior PS populations contributed to cardiomyocytes and other differentiated cells, suggesting that the cells at the stage of the PS formation are not yet completely committed toward the cardiac lineage in hESCs. Taken together, these findings would provide valuable opportunities to discuss the cellular and molecular mechanisms of the early cardiac developmental process in humans.

Poster Board Number: 3346

MICRORNA SIGNATURES IN SMALL MOLECULE INDUCED CARDIAC AND NEURAL LINEAGE-SPECIFICATION DIRECT FROM PLURIPOTENT HUMAN EMBRYONIC STEM CELLS

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The human embryonic stem cells (hESCs), derived from the inner cell mass (ICM) or epiblast of human blastocyst, are genetically stable with unlimited expansion ability and unrestricted plasticity, proffering a pluripotent reservoir for *in vitro* derivation of a large supply of disease-targeted human somatic cells that are restricted to the lineage in need of repair. One of the major challenges for both developmental studies and clinical translation has been how to channel the broad differentiation potential of pluripotent human stem cells to a desired phenotype efficiently and predictably. Although such cells can differentiate spontaneously *in vitro* into cells of all germ layers by going through a multi-lineage aggregate stage, only a small fraction of germ-layer-induced cells pursue a given lineage. In those hESC-derived aggregates, the simultaneous appearance of a substantial amount of widely divergent undesired cell types that may reside in three embryonic germ layers often makes the emergence of desired phenotypes not only inefficient, but uncontrollable and unreliable as well, hence, a high risk of tumorigenicity. To overcome these obstacles, in our study, we resolved the elements of a defined culture system necessary and sufficient for sustaining the epiblast pluripotence of hESCs, serving as a platform for *de novo* derivation of therapeutically-suitable pluripotent hESCs and effectively directing such hESCs uniformly towards clinically-relevant lineages by simple provision of small molecules. Employing the defined platform, we found that nicotinamide (NAM) and retinoic acid (RA) induced respective cardiac- and neural-lineage commitment direct from pluripotent hESCs that further progressed to beating cardiomyocytes and ventral neurons with drastic increases in efficiency. MicroRNAs (miRNAs) are emerging as important regulators of stem cell pluripotence and differentiation. To uncover key regulators, genome-scale miRNA profiling was used to identify novel sets of development-initiating small miRNAs upon small-molecule-induced cardiac- and neural-specification direct from the pluripotent stage. A unique set of pluripotence-associated miRNAs was down-regulated, while novel sets of distinct cardiac- and neural-driving miRNAs were up-regulated upon the induction of lineage specific differentiation from hESCs, including silencing of hsa-miR-302 family and a drastic expression increase of Hox miRNA hsa-miR-10 family upon RA exposure. Our study opens new dimensions of small molecule-mediated direct control and modulation of hESC fate when deriving an unlimited supply of clinically-relevant lineages from pluripotent cells for regenerative therapies.

Poster Board Number: 3348

IN-DEPTH PROTEOMIC AND PHOSPHOPROTEOMIC PROFILING AND COMPARATIVE SYSTEMS BIOLOGY ANALYSIS OF HUMAN PLURIPOTENCY AND NEURAL MULTIPOTENCY

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Defining 'stemness' in pluripotent and multipotent stem cells is crucial for comprehensive cell type characterization during early human development. Here, we demonstrate that human embryonic stem cells (hESCs) can be rapidly and completely converted into neural stem cells (NSCs) under feeder-free conditions by inhibition of TGF-β superfamily and WNT signaling. These

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pure populations of NSCs (97% PAX6+) were differentiated into various neuronal and glial phenotypes confirming their multipotent potential. By focusing on the transition from pluripotency to neuroectodermal multipotency, we delineated comparative (phospho)proteomic profiles from undifferentiated hESCs (OCT4+/PAX6-) vs. the earliest definitive human neural stem cells (NSCs; OCT4-/PAX6+) identifying a total of 12,904 proteins, 76.4% (9,964) of which were phosphorylated on a total of 59,680 sites. This is the largest comparative (phospho)proteomic dataset generated to date, allowing us to reveal numerous novel characteristics of pluripotent and multipotent stem cells: 1. Although the total (phospho)proteome overlap was 75.9% between these stem cell populations, only 25.7% of the localized phosphorylation sites were identified in both ESCs and NSCs suggesting that both stem cell states are predominantly defined by unique signatures on the posttranslational level, thus not detectable with commonly used microarray-based transcriptomic analysis. 2. Among the 27,964 precisely localized phosphosites, 55% were on serine (S) residues, 32% were on threonine (T) residues, and 13% were on tyrosine (Y) residues. These values were essentially the same in hESCs and NSCs; however, an unusually high percentage of Y phosphorylation sites was detected in stem cells relative to many other biological systems (typically ~1% Y). 3. We performed an unbiased and quantitative analysis of dynamic changes of proteins associated with pluripotency and epigenetic modifications (e.g. OCT4, SOX2, NANOG, c-MYC, Polycomb Group proteins, SWI/SNF chromatin remodeling complex, REST, DNMT3B, CTCF) including the identification of numerous novel phosphorylation sites. 4. Large-scale precision proteomics allowed comparative global pathway analyses of TGF-β superfamily and WNT signaling. 5. We detected surprisingly early expression and phosphorylation of proteins associated with later-stage neuronal function and neurological diseases (e.g. α-synuclein, amyloid precursor protein). A protein cluster map for Parkinson's disease, Alzheimer's disease, and Huntington's disease was generated as a resource for novel biochemical assays. Together, our findings shed new light on the proteome of human pluripotency and neural multipotency on the systems biology level and should facilitate the scalable production of defined neural phenotypes for developmental studies, disease modeling, and drug discovery.

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DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TOWARDS NEURAL ECTODERM USING SMALL MOLECULES

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Human embryonic stem (hES) cells have the potential to differentiate *in vitro* into cells and tissues of the three primary germ layers: endoderm, mesoderm and ectoderm. Therefore hES cells offer a great promise for cell replacement therapies. The molecular pathways and mechanisms underlying the differentiation of hES cells however have not been well understood. We have focused on the induction of hES cells to ectoderm, in particular anterior neural ectoderm (ANE). ANE cells have the potential to differentiate *in vitro* into cells of the brain, spinal cord, pituitary and eye. Based on knowledge from the early embryo development, where ANE development is regulated by native inhibitors of bone morphogenic protein (BMP) and Nodal/Activin signalling, most published protocols are demonstrating a crucial role SMAD signalling in the neural induction. The drawbacks of such protocols include the use of an embryoid body culture step (leading to a higher than desirable variability), as well as use of polypeptide secreted factors which are both expensive and have significant challenges in terms of GMP compliancy.
To increase the efficiency and reproducibility, as well as to understand the pathways and mechanisms involved in ANE induction, we have developed a monolayer cell culture methodology using xeno-free media to derive ANE cells from hES cells. In order to investigate the role of SMAD signalling in the neural induction we have tested a group of small molecules that are both selective and non selective Activin-receptor-like kinases (ALK) inhibitors. We have developed a protocol that requires only two small molecules LDN193189 (a BMP inhibitor) and SB431542 (ALK 4/5/7 inhibitor) for the efficient induction of ANE fate. This protocol enables the reproducible and efficient conversion of monolayer hES cells to ANE cells without the requirement of serum or polypeptide signalling factors in the culture medium.

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LONG TERM NEUROEPITHELIAL STEM CELLS CAPTURED DIRECTLY FROM THE DEVELOPING HUMAN BRAIN

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Introduction: Stably expandable neural stem cells in culture provide the unique opportunity to model neurodevelopment and human diseases that may involve neural progenitors in the developing brain such as congenital malformations and paediatric brain tumours. Recently, long term propagating stem cells that appear to represent neuroepithelial progenitors were derived from embryonic stem cells *in vitro*. We explored the possibility of deriving this neuroepithelial stem cell (NES) intermediate directly from human foetal brain as a first step towards elucidating the true relationship between NES cells and normal progenitors in the developing brain. Methods: The developing hindbrain was surgically dissected from Carnegie stage 14-23 (week 5 to 9) aborted human foetuses and dissociated into a cell suspension. Cells were cultured in monolayer with DMEM/F12 media with N2, B27, epidermal growth factor (EGF) and fibroblast growth factor (FGF2) supplements as previously described. Results: We found that neuroepithelial-like stem (NES) cells can be derived from dissected hindbrain tissue samples with reasonably high efficiency (7 out of 13 samples). All foetal NES cells were derived from Carnegie 14-18 (week 5-7) foetuses. Cells from older, Carnegie stage 19-23 (week 8-9) fetuses (2 samples) were reminiscent of more mature, radia-glia like neural stem (NS) cells. In similar fashion to pluripotent cell derived NES cells, the foetal derived NES cells exhibit rosette-like architectures in culture, express early neuroepithelial markers (Sox1, Pax6) and genes specific to neural rosette cells derived from embryonic stem cells (PLZF, Dach1). They can be passaged for more than 40 generations and retain the capacity to differentiate into neural and glial lineages. Foetal NES cells were found to retain expression of hindbrain regional identity markers (Gbx2, Krox20) after long term passaging, and display a gene expression profile that correlates with pluripotent cell derived NES cells. Conclusion and future directions: These findings suggest that NES cells are a common entity that can be derived from both pluripotent cells and the developing foetal hindbrain. With the advent of cell programming and the generation of patient-specific induced pluripotent stem cells (iPS cell), it is now possible to develop patient-specific NES cell models of neuroepithelial precursors. The generation of foetal derived NES cells provides evidence for a relationship between pluripotent cell-derived NES cells and neuroepithelial progenitors in the developing human brain, and provides an important benchmark for the future derivation of patient-specific iPS cell-derived NES cells. Future experiments will involve the *in utero* transplantation of foetal derived NES cells into foetal rat brain to determine if NES cells retain the capacity to integrate into the developing brain.

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PREDICTING TOXICITY ON DEVELOPING NEURAL CELLS BY USING THE HUMAN EMBRYONIC STEM CELL LINE H9

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Animal models used to predict prenatal toxicity often do not correlate to human response since the concordance between species is unsatisfying. Therefore it is urgent to develop more predictive tools in order to optimize the process of risk identification and characterisation. Human Embryonic Stem Cells (hESC) and their derivatives are currently scientifically discussed as the basis for toxicological tests including assays that aim to detect effects on early embryogenesis. For the detection of toxic effects on the developing nervous system, cells of the H9 cell line were differentiated into early neural precursor. Effects of nine toxicologically well defined compounds on this differentiation process were analyzed. In an initial step, the cytotoxicity of 6-Aminonicotinamide, 5-Fluorouracile, Lead acetate, Methylmercury, Methotrexate, Toluene, Retinoic acid, Valproic acid and Warfarin were defined. Resulting dose-response curves were used to determine critical dosages levels like the IC50 and the lowest non-cytotoxic concentration. These concentrations have been further analysed to assess changes of the gene expression profile after chemical treatments. The chemicals were clustered according to their toxicological responses in the differentiating cultures. The quality and reproducibility of the obtained data were ensured by the establishment of acceptance criteria defining successful independent experiments. In the present study we succeeded to develop an embryonic stem cell based test system that allows the detection of toxicological effects on differentiating embryonic stem cells. A first set of data deriving from a training set of chemicals allowed suggesting a prediction model which can be used to get a better understanding of effects of unknown compounds. Further work will be needed to refine this model and challenge it with additional xenobiotics to fully evaluate its predictive capacity.

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PRODUCTION OF HIGH PURITY NEURONAL AND OLIGODENDROCYTE PROGENITOR CELL POPULATIONS FROM HUMAN IPS AND BLASTOCYST-DERIVED HES CELL LINES

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Background: The discovery that human somatic cells can be reprogrammed to induced pluripotent stem cells (hiPSCs) which closely resemble human embryonic stem cells (hESCs) garnered tremendous excitement. While iPS lines pass gross tests of pluripotency, more stringent characterization indicates that most lines are partially rather than completely reprogrammed. The objective of our work was to, 1) determine the degree of equivalence between three human iPS and two blastocyst-derived human ESC lines and 2) determine whether differences have functional implications for the ability of lines to produce neuronal and oligodendrocyte progenitor lines via clinically relevant high purity differentiation protocols. Methodology/ Principal Findings: Two blastocyst-derived hESC lines (H7 and CSC-14) and three hiPSC lines derived from adult human dermal fibroblasts were exposed to identical expansion conditions followed by differentiation into neuronal and oligodendrocyte progenitor cells. Proliferation rates and gross morphology were recorded during expansion. During stem cell expansion, all lines had similar rates of proliferation and gross morphology. Yield and purity of differentiated products were determined via cell counts and ICC profiling. ICC profiling indicated that both hESC lines and two of the three hiPSC lines produced differentiated products with a low percentage of contaminating cell populations. These two iPS lines matured faster than the hESCs. The third iPS line produced a very low yield of differentiated product, consisting primarily of undifferentiated neural cells. Despite the recently published differences between hiPSCs and hESCs, properly screened iPS lines are capable of producing differentiated products of purity sufficient for clinical applications and should be tested *in vivo* to determine if differentiated products have functional equivalence to those produced from hESC lines.

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DIRECTING HUMAN EMBRYONIC STEM CELLS TO FUNCTIONAL HEPATOCYTE-LIKE CELLS USING CONDITIONED MEDIA FROM A HUMAN HEPATOMA CELL LINE

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Hepatocytes derived from human embryonic stem cells (hESC) promises to address issues in drug discovery and treatment of liver diseases. We have developed a novel method to efficiently differentiate three different hESC lines; BG01, Relicell®hES1 and Relicell®hES2 into functional hepatocytes. To establish the protocol, we reasoned that conditioned media of HepG2, a human hepatoma cell line, may contain pro-hepatic factors and priming the hESCs with this media in the early stages can initiate hepatic commitment. Here we have attempted to recapitulate the *in vivo* liver development by using conditioned media from HepG2 cell line, supplemented with specific growth factors involved in liver development such as aFGF, HGF, oncostatin, dexamethasone and EGF for stage specific hepatic differentiation. For further maturation the differentiated hepatocytes were cultured in a defined hepatocyte maintenance media. The differentiated cells generated by this protocol exhibited the typical polygonal morphology of hepatocytes and expressed genes such as HNF4a, AFP and ALBUMIN as seen by real time PCR. Importantly these cells also expressed Phase I (CYP3A4) and Phase II (GST A1) drug metabolism enzymes which are responsible for metabolizing most of the chemical compounds. The expression of markers involved in the various stages of hepatocyte development was examined by immunofluorescence and FACS. In the final stage, 70% of the differentiated cells expressed hepatic lineage markers like albumin, CK8/18 and EpCAM. Furthermore, the functional assays confirmed that the differentiated cells were able to carry out hepatocyte functions such as production of urea and glycogen storage. These results suggest that our unique protocol is successful in generating functional hepatocytes and is also efficient as shown with three genetically different hESC lines. These differentiated cells can provide *in vitro* drug toxicity testing platform in the future.

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EFFICIENT GENERATION OF FUNCTIONAL HEPATOCYTES FROM HUMAN EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS BY HNF4α TRANSDUCTION

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Induced hepatocytes from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are expected to be utilized as a tool for screening for cytotoxicity in the early phase of pharmaceutical development. Although conventional methods such as growth factor-mediated hepatic differentiation are performed, they only lead to generation of a heterogeneous hepatocyte population. To improve this, transduction of transcription factors is performed to promote hepatic differentiation by using adenovirus (Ad) vectors. We have recently reported that hepatic commitment is promoted by transduction of SOX17 and HEX gene, although these induced hepatocytes were not enough matured for drug screening. To promote hepatic maturation, we utilized transduction of the hepatocyte nuclear factor 4α (HNF4α) gene, which is known as a master regulator of liver-specific gene expression, in addition to transduction of SOX17 and HEX gene. Overexpression of HNF4α in hepatoblasts resulted in the upregulation of the gene expression level of the epithelial markers and the mature hepatic markers related to drug metabolism including cytochrome P450 enzymes. The flow cytometry analysis showed that HNF4α transduction led to almost homogeneous hepatocyte population; the differentiation efficacy based on the CYPs, ASGR1, or ALB expression levels was approximately 80%. The Ad-HNF4α-transduced cells exhibited an abundant characteristics of hepatocytes; uptake of LDL, uptake and excretion of ICG, and storage of glycogen. Furthermore, induced hepatocytes could catalyze the toxication of several compounds such as Troglitazone, Acetaminophen, Cyclophosphamide, and Carbamazepine. We also found that HNF4α overexpression in hepatoblasts promoted hepatic differentiation by activating mesenchymal to epithelial transition (MET). In summary, the findings described here demonstrate that transduction of HNF4α could promote hepatic differentiation from human ESC-derived hepatoblasts by activating MET. In the present study, both human ESCs and iPSCs (3 lines) were used and all cell lines showed efficient hepatic maturation, indicating that our protocol would be a universal tool for cell line-independent differentiation into functional hepatocytes. Moreover, human ESC- or iPSC-derived hepatocytes would a valuable tool to predict drug toxicity because induced hepatocytes have ability to catalyze the toxication of several compounds as primary human hepatocytes.

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EFFECT OF FGF7 TO DIRECTED DIFFERENTIATION OF HUMAN ESCS INTO AMYLASE-POSITIVE CELLS

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The pancreatic exocrine cells secrete digestive enzymes, which comprise major enzymes involved in the digestion of foodstuffs. However, cultivation of functional pancreatic cells isolated from adult mammalian pancreas remains difficult to achieve. Recently, embryonic stem (ES) cells have received much attention as the resource of cells to evaluate activity of drug and food as well as cell transplantation therapy. In addition, it was reported that the effect of cholecystokinin, the most important mediator of postprandial pancreatic exocrine enzyme output is species-specific on pancreas. Therefore, it is significant to use human cells to prediction experiment of human digestive reaction *in vitro*. The goal of our study is to construct digestive system with human ES cell-derived pancreatic cells which could imitate normal human tissues *in vitro*. In this study, we investigated whether human ES cells can be induced into pancreatic exocrine cells without embryoid body (EB) formation. Initially, undifferentiated human ES cells (KhES-3) were cultured with Activin A and Wnt3a for the differentiation of definitive endoderm. Then, the cells were treated with retinoic acid for 3 days, followed by treatment with FGF7, GLP-1 and Nicotinamide. As a result, amylase-positive cells were effectively induced by FGF7, and PDX1-positive cells, a marker for pancreatic progenitor, were localized in proximity to amylase-positive population. Additionally the expression levels of pancreatic endodermal markers such as Foxa2 and Pdx1 were up-regulated in previous step and these amylase-positive cells were double-positive with carboxypeptidase A. All these results suggested that the ESC-derived amylase-positive cells were regarded as pancreatic exocrine cells. The present study indicates reproducible induction of pancreatic exocrine cells from human ES cells. Our induction protocol for pancreatic exocrine cells provides an insight into the use of human ES cells for imitating digestive systems in drug and food development. The physiological analysis of the differentiated pancreatic exocrine cells and improvement of differentiation efficiency is now ongoing. (Yoshie, Mogi and Sasaki, who had been permitted to culture human ES cells by the government, performed all steps of culturing of human ES cells.)

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CHARACTERISATION OF PANCREATIC PROGENITORS AND GUT ENDODERM DERIVATIVES DERIVED FROM HUMAN EMBRYONIC STEM CELLS

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The directed differentiation of human embryonic stem cells (HESCs) into functional pancreatic beta cells has attracted much interest as a potential treatment for diabetes. Several groups have demonstrated the efficient *in vitro* production of pancreatic progenitor cells or immature beta cells from HESCs. However, to date, no published report has described the production of these cell types to homogeneity. Here, we describe a simple, chemically-defined protocol that yields ~ 60 % HESC-derived PDX1+ pancreatic progenitors in adherent culture. This protocol is reliant upon the synergistic activity of combined Activin A and BMP4 treatment between days 0 and 3 of differentiation, which results in rapid downregulation of pluripotency

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genes and robust formation of both definitive endoderm (DE) and PDX1+ cells. Q-PCR, immunostaining and western blot analyses show that many of the signature pancreatic endoderm genes in addition to PDX1 such as HNF6, NGN3, NEUROD and NKX2.2 are increasingly expressed between days 12 and 17. Similar to the highly cited study from Novocell / ViaCyte, we have initiated transplantation experiments into immuno-deficient mice to characterise whether PDX1+ progenitors derived under these conditions develop into functional beta cells *in vivo* and maintain euglycemia. We also investigated the identity of the remaining ~ 40 % cells by marker gene analysis assuming that this population likely contains other DE derivatives. Not surprisingly, we detect expression of stomach (RAE1 and HOXA3), liver (AFP and ALB) as well as the small intestine (DPP4, CDX2, HOXA4 and HOXA5) marker genes. Microarray experiments at selected time points confirm the upregulation of numerous genes involved in gut endoderm formation. We are extending these observations by FACS and immunohistochemistry. Overall, our HESC-derived PDX1+ pancreatic progenitors develop with other gut endoderm derivatives and are thus developmentally relevant. Future work includes the use of this robust differentiation protocol to study the role of novel pancreatic genes.

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DEVELOPMENT OF 3D CULTURE METHODS FOR THE HEPATOCYTIC DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS

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Hepatocytes derived from human pluripotent stem cells (hPSC) could provide a defined and renewable source of human cells relevant for cell replacement therapies and toxicology studies. Moreover, patient cell-derived iPS cells contain the genotype responsible for the human disease and so provide a possibility for experimental disease modeling and therapeutic exploration. Differentiation of definitive endoderm (DE) is a crucial first step in generating hepatocytes. 3D matrices are expected to provide a more natural microenvironment for the maturation of the differentiating DE cells as compared with 2D culture. We have established a modified method for efficient induction of DE from hPSCs. We routinely observe up to 95% FOXA2/Sox17/CXCR4 positive cells. The DE- cells were further differentiated into hepatic progenitors in the presence of DMSO and then matured for 7 days with HGF and Oncostatin M into hepatocyte like cells. The cell population generated in this culture condition showed a morphological resemblance to human hepatocytes and expressed hepatocyte specific markers at RNA and protein level. The cells were also able to secrete albumin. In order to identify the optimal niche for differentiation, we next studied the effect of various purified ECM proteins. Based on immunocytochemistry and albumin secretion, collagen III and fibronectin showed the best capacity to support maturation of DE-cells towards hepatocyte like cells. According to this, collagen III and fibronectin are candidates as supporting components in otherwise inert 3D-culture matrix. Several commercial matrices intended for 3D culture were tested, of which HydroMatrix (Sigma), HyStem-C (Sigma) and ExtraCel (TebuBio) have been studied in more detail. DE-cells differentiated most efficiently into hepatocyte like cells in ExtraCel based on highest albumin secretion, highest relative albumin gene expression and strongest Cyp3A4 and albumin immunoreactivity. However, during a 21-day differentiation protocol, the overall level of hepatocytic differentiation was not significantly higher in 3D than in standard 2D culture. Longer maturation times are likely needed to obtain a higher degree of differentiation in the 3D environment.

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DERIVATION OF FUNCTIONAL HEPATOCYTE LIKE CELLS FROM HUMAN PLURIPOTENT STEM CELLS

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Human embryonic and induced pluripotent stem cells represent novel sources of human hepatocytes for transplantation, for drug toxicity screens and for basic developmental biology studies. Although several studies have already demonstrated that it is possible to direct the differentiation of human pluripotent stem cells (hPSCs) to the hepatic lineage, generating functionally differentiated hepatocytes remains a major challenge. To be able to efficiently and reproducibly generate hepatic lineage from hPSCs, it is essential to recapitulate the critical induction events of liver development *in vitro*, including endoderm induction, hepatic specification and hepatocyte maturation. Efficient endoderm induction is the first key step in generating cells of the hepatic lineage from hPSCs. We have found that the combination of activin A, Wnt3A and bFGF is required to induce an embryoid body (EB) population that consists of greater than 90% endoderm as measured by expression of the cell surface markers as c-KIT and CXCR-4 and the transcription factor SOX17+. For hepatic specification, the EB cells are dissociated and plated as a monolayer in the presence of activin for an additional 2 days and then in the presence of BMP-4 and bFGF for 6 days. Sustained activin-induction is important for efficient hepatic development and the optimal timing of this step varies among different hPSCs. Following hepatic induction, the population is maintained as a monolayer in the presence of HGF, Dex and OSM for 12 days to generate a hepatoblast population characterized by expression of AFP, ALB and CK19. To induce hepatic maturation, the hepatoblast stage cells are harvested and cultured for an additional 18 days as aggregates. Beyond 40 days of differentiation, the aggregates consist of greater than 90% albumin+ cells, 60 % Asialoglycoprotein receptor-1 positive cells and express levels of albumin, G6Pase, and TAT comparable to those of found in the adult liver. Analyses of the aggregate cells for CYP1A2 and UGT activity revealed levels similar to those found in primary human hepatocytes. Taken together, these findings show that efficient endoderm induction of hPSCs following by appropriate specification and maturation cues results in the emergence of hepatocyte-like populations that display characteristics of primary human hepatocytes.

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TEMPORAL REGULATION OF HNF4α OVER-EXPRESSION ALTERS ANTERIOR-POSTERIOR FATE DURING HUMAN EMBRYONIC STEM CELL (HESC) DIFFERENTIATION

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hESC-derived definitive endoderm (DE) tissues such as liver and intestine are of great interest in regenerative medicine however, DE differentiation pathways are poorly understood. We have previously demonstrated that SOX17 over-expression in hESCs results in an anterior DE progenitor population providing an *in vitro* model to elucidate the molecular mechanisms that specify

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DE. *In vivo*, the developing embryo undergoes morphological changes as the DE differentiates into the primitive gut tube. The primitive gut tube is divided into organ domains marked by organ-specific transcription factor expression. The nuclear receptor transcription factor HNF4α is expressed in the intestine, liver, pancreas and stomach throughout organogenesis and is important for differentiation and maturation of these cell types. In fibroblasts, HNF4α over-expression induces intestinal gene expression. The aim of this work is to use HNF4α-hESCs as a tool to investigate the molecular regulation of primitive gut tube development and differentiation. To generate HNF4α-hESCs, HES2 hESCs were transfected with a PiggyBac transposon containing HNF4α under the control of the tetO2 tetracycline/doxycycline inducible promoter. Following 48 hours of HNF4α over-expression, HNF4α-hESCs undergo morphological changes resulting in a flattened morphology and a high cytoplasm to nucleus ratio. Gene and protein expression profiling of HNF4α-hESCs reveals an induction of the HNF4α intestinal target CDX2 as well as PDX1 and SOX2, consistent with posterior gut identity *in vivo*. HNF4α over-expression downregulates pluripotency genes OCT4 and NANOG and does not induce expression of extraembryonic endoderm genes SOX7, LAMB1 and AFP. To determine whether the temporal over-expression of HNF4α can induce liver specific function during hESC differentiation, HNF4α was induced at three stages of hepatocyte differentiation, DE specification, hepatic specification and hepatic maturation. Induction of HNF4α following DE specification produced inefficient hepatic differentiation and induction of the posterior DE marker CDX2. However, when HNF4α is induced following hepatic specification or maturation, hepatic differentiation is more efficient compared to non-induced controls as demonstrated by CYP1A2 and CYP3A7 expression. Additionally, the posterior DE gene CDX2 was not induced. These preliminary experiments demonstrate that the temporal regulation of HNF4α during hESC differentiation can alter anterior-posterior DE fate.

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THE GENERATION AND ISOLATION OF NKX2.1 EXPRESSING POPULATIONS FROM HUMAN PLURIPOTENT STEM CELLS, AND THEIR ABILITY TO PRODUCE LUNG EPITHELIUM.

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Although hPSCs are maintained *in vitro*, they still respond to the core developmental programs identified in the mouse embryo that control gastrulation, the generation of definitive endoderm (DE) and subsequent differentiation into the gut tube. Foregut endoderm is the precursor tissue that gives rise to the lung buds, which in turn are delineated by the expression of Nkx2.1. Work in our lab has examined the correlation of expression of the human orthologs of genes known to be important for the specification of the DE (i.e. EOMES, SOX17, FOXA2) using Wnt3a, high activin A, and low serum. We have found that NKX2.1 is expressed early during the induction of DE, and co-expressed with other transcription factors (TFs) important for DE and subsequent foregut specification, including FOXA2. We have made a fluorescent reporter for NKX2.1 that enables us to detect and isolate cells expressing NKX2.1 after DE-induction, and have confirmed these populations are enriched for genes expressed in the DE. We have also utilized TF gain of function to further evaluate the role of NKX2.1 in the specification of foregut and lung progenitors. We have combined these tools with an *in vitro* air-liquid interface culture system to investigate the role of NKX2.1 in the generation of human lung progenitors. Our study provides insight into the efficient generation of lung progenitors from hPSCs for future use in tissue replacement therapies and drug discovery.

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RAPAMYCIN IMPROVES THE EFFICIENCY OF DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO DEFINITIVE ENDODERM

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Definitive endoderm (DE) formation is the most important stage that all endodermal organs pass through during their development. So, *in vitro* production of definitive endoderm is one of the important issues in stem cell related differentiation studies. Introducing more defined and efficient methods for differentiation of human embryonic stem cells (hESCs) to the DE can help to produce more efficient endoderm derivatives for therapeutic applications. We selected chemicals Rapamycin, Stauprimide, CHIR, NSC-308848, LY294002, Cymarin and IDE1 and also growth factor Activin A. During the differentiation induction different combinations of these inducers applied on hESCs and then DE specific markers investigated in both RNA and protein expression levels. The control group was Wnt and Activin A for the 1st day and activin A for the next 3 days. According to the real time RT-PCR results, one of our inductive groups composed of Rapamycin (200 nM, 1st day) and Activin A (50 ng/ml, the next 3 days) increased the SOX17 and FOXA2 expression levels significantly more than our control group. The flowcytometric and immunofluorescent staining results were also in agreement with the gene expression results. These findings may help to design more efficient chemically defined protocols for DE induction of hESCs and also may lead to better understanding of the different signaling networks involve in DE differentiation.

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TRANSLATING CELL BIOLOGY INTO RATIONAL BIOPROCESS DESIGN: EFFICIENT, SCALABLE PRODUCTION OF DEFINITIVE ENDODERM FROM HUMAN PLURIPOTENT STEM CELLS AS VALIDATION OF A NOVEL STRATEGY

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Capitalizing on the therapeutic potential of human pluripotent stem cells (hPSC) will require translation of bench-scale proof-of-concept experiments to robust and scalable production bioprocesses. Few such technologies are currently available, and rational bioprocess design strategies required to develop them are under-explored. We will present the application of a predictive strategy employing cell- and molecular-level analysis of rate limiting steps to produce definitive endoderm (DE) progenitors in scalable, microwell-aggregation-based directed differentiation technologies. We have defined a bioprocess optimization parameter (L; targeted cell Loss) and, with quantitative cell division tracking and fate monitoring of hPSC expansion and differentiation to DE, identify and overcome key suspension bioprocess bottlenecks. Targeting these parameters in a cell-line specific manner en-

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abled adherent-equivalent growth rates of hPSCs in feeder- and matrix-free defined-medium suspension culture. Taking these suspension expanded cells we then demonstrated that predominantly instructive (as opposed to selective) differentiation mechanisms underlied a subsequent 18-fold expansion of cells during directed hPSC differentiation to high-purity DE (C-KIT+/CXCR4+/CD31- by flow cytometry with significantly upregulated transcription of SOX17, FOXA2, Cerberus and GATA3 but not PDGFRA, KDR, SOX7, FOXA3 or SOX1;) competent for further differentiation towards pancreatic (INS, PDX-1, SOX9-expressing) and hepatic (AFP, Albumin, Cyp3A4-expressing) fates. Our coupled expansion and differentiation systems employ only defined culture components, and permit the generation of over 109 CKIT+ CXCR4+ double positive cells from 1.5 x 104 input cells in 22 days, employing only 4,000 cm2 of combined culture surface (the area of a circle roughly the size of a bicycle wheel, less than a single 10-deck cell factory), providing a solid foundation for future preclinical and clinical studies. The aggregates themselves are confined to unit cells with dimensions 400 x 400 x 283 microns, giving final densities exceeding 8 x 106 cells per mL. To facilitate the rapid incorporation of additional future process and culture improvements, we focused on scale-independent solutions and "open-access" approaches that are easily replicated, improved, and extended upon without requiring unconventional technical capabilities. By quantitatively assessing such key, cell-line specific parameters as cycling rate and initial survival we are able to prospectively specify optimal induction conditions to guide the optimized production of target cells from other cell lines in our scale-free directed differentiation system. To our knowledge, this is the first application of this approach to the production of differentiated cells from hPSC, and provides a generally applicable template for the translation of bench-scale experimental work into robust cell production for industrial, preclinical and clinical applications.

Poster Board Number: 3378

DERIVATION OF STROMAL (MESENCHYMAL) STEM-LIKE CELLS FROM HUMAN EMBRYONIC STEM CELLS IN SERUM FREE ENVIRONMENT

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Derivation of bone forming cells from human embryonic stem cells (hESC) is a potentially useful tool for clinical applications. However, there is no standard protocol for differentiating of hESC into osteoblastic cells. In addition, when implanted *in vivo* as undifferentiated cells, hESC forms teratomas with a mixture of various mesodermal, endodermal and ectodermal tissues and thus limiting their clinical use. The aim of this study was to design a protocol for directing the differentiation of hESC into osteoblastic cells and test the ability to form bone *in vivo*. We cultured hESC in absence of serum and mouse feeder layer and in suspension as 3D-embryoid bodies (hEB) aggregates. During a time course of 20 days, the hEBs became enriched for cells expressing mesenchymal stem cell (MSC) markers: CD29, CD44, CD63, CD56, CD71, CD73, CD105, CD106 and CD166 as revealed by immunohistochemistry and the number of positive cells increased with time during 20 day period. In order to test the functional ability of hESC-derived MSC, we implanted day 20 hEBs mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) as an osteoconductive scaffold, subcutaneously in immune deficient mice. Bone and cartilage as well as fibrous tissue were detectable, after 8 weeks of implantation. There was no evidence for differentiation to other non-mesodermal tissues. This is in contrast to undifferentiated hESC that formed a teratoma with a mixture of endodermal, mesodermal and ectodermal tissues. Also, hEB implanted in absence of HA/TCP formed cysts containing glandular, fibrous and muscle-like tissues. Our study demonstrates the feasibility of enriching EBs for osteogenic cells under serum-free culture conditions.

Poster Board Number: 3380

THE CELL CYCLE AND MESODERM INDUCTION IN HUMAN ES CELLS

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Experiments in model organisms and embryonic stem cells have shown that differentiation is associated with a lengthening of the cell cycle. We hypothesise that passage through the cell cycle, in addition to its gradual lengthening, is required for efficient transcription of early differentiation-associated transcription factors. Here we focus on mesoderm as a model for differentiation and examine the role of the cell cycle in the induction of the early mesoderm-associated transcription factor Brachyury (T). Mesoderm is an embryonic germ layer from which tissues such as bone, blood vessels, the spleen, the kidneys, cardiac and skeletal muscle are formed. Therefore there is a high demand for efficient *in vitro* protocols to induce human embryonic stem cells (hESCs) to form mesoderm. Upon mesoderm induction with a combination of FGF2, an inhibitor of PI3K (LY294002) and BMP4 (collectively known as the FLyB protocol), H9 hESCs cease proliferation but still differentiate efficiently. In contrast, using another mesoderm induction protocol comprising an inhibitor of GSK3b (CHIR99021), FGF2 and BMP4 (also known as the ChFB protocol), hESCs continue to both proliferate and differentiate. We then exposed asynchronous H9 hESCs to FLyB and ChFB for various lengths of time within one ES cell cycle, sorted them by DNA content and performed quantitative real-time PCR to examine T transcript accumulation by cell cycle phase. Interestingly, there was a statistically significant cell cycle-associated accumulation of T transcript at 12h using the FLyB protocol. Other mesoderm-associated, pluripotency and housekeeping genes did not show the same expression pattern. Disrupting cell cycle progression using aphidicolin blocked the accumulation of T using the FLyB protocol but not with the ChFB protocol. Paradoxically, these findings imply that passage through S phase is responsible for the bulk of T transcription using the FLyB protocol, but not ChFB. Our results indicate that induction of hESCs into early mesoderm progenitors using FlyB increases T expression but that the cell cycle is a major barrier to the long-term maintenance of differentiated cells.

Poster Board Number: 3382

APELIN PROMOTES GROWTH OF HEMATOPOIETIC COLONIES FROM HUMAN EMBRYONIC STEM CELLS

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The *in vitro* differentiation of human embryonic stem cells (hESCs) recapitulates aspects of embryonic development, whereby cells destined to differentiate into mesoderm or endoderm pass through a primitive steak-like stage. We have used gene targeting by homologous recombination to generate a hESC line that expresses a GFP reporter gene from the MIXL1 locus (MIXL1GFP/W), which results in transient GFP expression in differentiating mesendodermal cells that will later adopt mesoderm or endoderm fates. We have utilized transcriptional profiling of differentiating MIXL1 GFP/W hESCs to identify genes expressed during early mesoderm differentiation from hESCs. Transcriptional profiling of flow sorted mesendodermal cell populations revealed that MIXL1-GFP+ cells transiently expressed high levels of the AGTRL1 (hAPJ receptor) gene. Prior literature has linked hAPJ and its ligand, Apelin, to cardiovascular development and angiogenesis. In the light of its

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expression in early primitive streak-like cells, we have examined the role of Apelin/APJ signaling on hematopoietic differentiation *in vitro*. We identified a reproducible augmentation in hematopoietic colony formation following the supplementation of the culture with a bioactive Apelin peptide during mesodermal differentiation and during hematopoietic blast colony formation. With the use of a synthesized fluorescein conjugated Apelin peptide we were able to isolate APJ+ population after primitive streak induction and confirmed the enrichment of hematopoietic colony forming cells within the APJ+ population. Transcriptional profiling of mesoderm differentiation culture revealed an increase in fetal globulin genes following day2 treatment of Apelin peptide as well as the accelerated upregulation of other developmental gene groups. Apelin peptide, together with VEGF also synergistically enhanced the proliferation and hematopoietic colony frequency of KDR+/CD34+ endothelial progenitors *in vitro*.

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DIRECTED DIFFERENTIATION OF HUMAN ESC AND IPSC TOWARD THE MEGAKARYOCYTE LINEAGE

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The generation of megakaryocytes from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) provides a model system for studying the earliest stages of lineage commitment *in vitro* as well as supplying an unlimited source of cells for functional applications. With an embryoid body (EB)-based protocol, in serum-free media we are able to generate hematopoietic populations with megakaryocyte potential from hESC and iPSC lines. We utilized a three step EB-based protocol that involves the formation of a primitive-streak-like (PS) population, the induction and specification of mesoderm, and the expansion of the megakaryocyte lineage. As shown by Kennedy et al. (Blood 109:2679, 2007), the primitive erythroid and macrophage lineages are generated within the developing EBs following the establishment of the hemangioblast and are detected in a population that co-expresses KDR and CD31. By using BMP-4, VEGF, bFGF, and hematopoietic cytokines, kinetic analyses of this population of cells shows that KDR expression diminishes over time followed by the emergence of a KDR-CD31+ population. This population co-expresses CD41 (αIIb) and CD235 (glycophorin A), markers on megakaryocyte-erythroid progenitor (MEP) cells. Between days 8 and 10 of differentiation, this MEP population is released from the EBs as single cells resulting in a homogenous population of MEPs that does not require cell sorting and results in a yield of one MEP per input hESC or iPSC. Co-culture of this MEP population on the stromal cell line OP9 for 3-7 days in the presence of cytokines, including TPO, promotes further maturation toward the megakaryocyte lineage as defined by the surface expression of CD42b (GPIbα) and CD42a (GPIX) expression, the appearance of multinucleated cells, the upregulation of the expression of genes associated with megakaryocyte commitment including PF4, and functional properties including PAC-1 and soluble fibrinogen binding following agonist stimulation. Ploidy analyses show that this CD41+CD42+ population is predominantly 2N. Various conditions are being tested to increase ploidy levels in these cells followed by analyses of platelet production *in vitro* and *in vivo*. These studies demonstrate the power of using hESC and iPSC for the study of hematopoiesis, especially for megakaryocytes which are an extremely rare and a difficult cell type to study.

Poster Board Number: 3386

DIRECT DIFFERENTIATION OF ATRIAL AND VNETRUCULAR MYOCYTES FROM HUMAN EMBRYONIC STEM CELLS

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Although myocyte cell transplantation studies have suggested a promising therapeutic potential for myocardial infarction, a major obstacle to the development of clinical therapies for myocardial repair is the difficulties associated with obtaining relatively homogeneous ventricular myocytes for transplantation. Human embryonic stem cells (hESCs) are a promising source of cardiomyocytes. Our previous study demonstrated that retinoid signalling regulates the fate specification of atrial versus ventricular myocytes during cardiac differentiation of hESCs. We found that both Noggin and the pan-retinoic acid receptor antagonist BMS-189453 (RAi) significantly increased the cardiac differentiation efficiency of hESCs. Our results showed that the expression levels of the ventricular-specific gene IRX-4 were radically elevated in Noggin+RAi-treated cultures. MLC-2v, another ventricular-specific marker, was expressed in the majority of the cardiomyocytes in Noggin+RAi-treated cultures, but not in the cardiomyocytes of Noggin+RA-treated cultures. Flow cytometry analysis and electrophysiological studies indicated that with 64.7±0.88% (mean ±s.e.m) cardiac differentiation efficiency, 83% of the cardiomyocytes in Noggin+RAi-treated cultures had embryonic ventricular-like action potentials (APs). With 50.7±1.76% cardiac differentiation efficiency, 94% of the cardiomyocytes in Noggin+RA-treated cultures had embryonic atrial-like APs which demonstrate that retinoid signalling specifies the atrial versus ventricular differentiation of hESCs. Currently, we are investigating the underline mechanisms that retinoid signalling regulating atrial vs. ventricular myocytes specification with high though put genetic analysis approaches.

Poster Board Number: 3388

TPO-R AGONIST, ELTROMBOPAG, PROMOTES DIFFERENTIATION OF HUMAN ES CELLS INTO MEGAKARYOCYTES AND PLATELETS IN PROTOCOLS DERIVED BY MULTIPLEXED COMBICULT TECHNOLOGY

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Human embryonic stem (hES) cell derived hematopoietic progenitors represent a renewable source of material for use in screening and evaluation assays in the search for novel therapeutic and regenerative drugs. Here we describe the discovery of novel serum-free feeder-free protocols that direct differentiation of hES cells to functionally active megakaryocytes and platelets. Additionally these protocols feature the replacement of commonly used cytokines with small molecule bioactives. Novel differentiation protocols were discovered using a high throughput screen called CombiCultTM (Combinatorial Cell Culture) that is capable of multiplexing very large numbers of cell differentiation protocols to identify critical combinations that result in high efficiency differentiation to a given phenotype. Human ES cells were grown on microcarriers and shuffled randomly through 40 different culture conditions, with concomitant labeling of the beads using nanomaterial tags. Ten thousand distinct protocols were sampled in one experiment. Following screening to identify beads bearing cells positive for the megakaryocyte specific marker cd41a and analysis of the tags to deduce cell culture history,

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90 novel protocols were identified. In a number of protocols efficient differentiation of hES cells into megakaryocytes and platelets was achieved through the use of small molecule bioactives such as the TPO receptor agonist eltrombopag, replacing lineage specific cytokines at different stages of differentiation and platelet formation. Cell populations produced by eight highly efficient protocols were further characterized for surface marker expression and functional properties.

Poster Board Number: 3390

ENHANCED INDUCTION OF HUMAN PLURIPOTENT STEM CELLS INTO PROLIFERATIVE AND FUNCTIONAL CYTOTROPHOBLAST

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During early human embryonic development, the trophectoderm (outer layer of the blastocyst) develops into extraembryonic ectoderm, which subsequently forms the epithelial compartment of the placenta, the trophoblast. The placenta broadly consists of two types of trophoblast: (a) villous trophoblast, including the multinucleated, βhCG-secreting, syncytiotrophoblast, and (b) extravillous trophoblast, an invasive tissue found at the maternal-fetal interface, which express cell surface marker HLA-G. It has been postulated that villous and extravillous trophoblast arise from a common precursor termed the 'Trophoblast Stem' (TS) cell, which resides within the proliferative cytotrophoblast compartment. Cytotrophoblast forms a continuous layer in placenta during the first trimester, but, as gestation proceeds this layer becomes fragmented, possibly due to depletion of endogenous TS cells. In chorionic villi, cytotrophoblast cells fuse to form the syncytiotrophoblast. Independently in anchoring villi at the maternal-fetal interface, the cell column of cytotrophoblast gives rise to invasive extravillous trophoblast. Development of an *in vitro* model for human trophoblast differentiation has met with several challenges. While BMP4 treatment of human pluripotent stem cells (both hES and hiPS cells) induces trophoblast differentiation within days, a stable self-renewing population has yet to be isolated. Instead cultures terminally differentiate with a bias toward syncytiotrophoblast, based on morphology and βhCG secretion. Here, we use a novel set of proliferative trophoblast markers, including CDX2 and TP63, to establish culture conditions for formation and maintenance of putative human TS cells. We find that presence of basic Fibroblast Growth Factor (bFGF) and physiologic oxygen tension (5% O2) enhance expression of trophoblast markers between 3-5 days in BMP4-supplemented media. Additionally, we compare such *in vitro*-derived trophoblast cultures to primary cytotrophoblast representative of first, second, or third trimester placenta, and find that the established culture based system most closely resembles first trimester cytotrophoblast - both in terms of global gene expression profile and functionality. We are currently in the process of identifying surface markers for culture-derived proliferative trophoblast and plan to use these markers to further enrich and optimize culture conditions for these cells. Our ultimate goal is to develop *in vitro* models useful in interrogating early placental development and related pregnancy disorders and to produce a novel platform for drug testing and other *in utero* therapeutics.

Poster Board Number: 3392

OPTIMIZING HEMATOPOIETIC DIFFERENTIATION FROM HUMAN EMBRYONIC STEM CELLS USING A HIGH-THROUGHPUT SCREENING ASSAY

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The differentiation of pluripotent stem cells to derivative cell types is of great interest for both basic science research and for the development of cell replacement therapies. We have developed a 96-well plate, 4-color flow cytometry based screening system to optimize embryoid body (EB) serum-free differentiation of human embryonic stem cells (hESCs) to desired lineages. We induced efficient differentiation to the three primary germ lineages (endoderm, mesoderm, and ectoderm) and successfully distinguished cell types by using specified combinations of four surface markers. Endoderm (CXCR4+KDR-), mesoderm (KDR+SSEA-3-), and ectoderm (SSEA-3+NCAM+) differentiation yields for H9 cells were 80 ± 11%, 78 ± 7%, and 41 ± 9%, respectively. Additionally, we screened for the effect of manipulating concentrations of four cytokines upon hematopoietic differentiation. In sequential screens, the concentrations of BMP4, VEGF, bFGF, and FLT3 ligand were varied at specified time points over six days of EB differentiation of H9 hESCs. Initially, EBs were seeded at day 0 and supplemented with specified concentrations of BMP4 (0-50 ng/mL) and VEGF (0-100 ng/mL). At day 3, cells were given medium containing bFGF and VEGF with no BMP4. At day 6, cells were harvested and analyzed using 96-well flow cytometry. VEGF induced proportional increases in both CD34 and CD31 expression, with 100 ng/mL inducing CD34 and CD31 expression in 30-40% of cells. 70-90% of cells consistently expressed KDR, with greater concentrations of BMP4 inducing moderately increased KDR expression. Based off of this data, EBs were seeded at day 0 and supplemented with 10 ng/mL BMP4 and 50 ng/mL VEGF. At day 2, EBs were supplemented with medium containing 50 ng/mL VEGF, bFGF (0-10 ng/mL), and FLT3 ligand (0-40 ng/mL). Flow cytometry was performed at days 4 and 6. Mid-range FLT3 ligand concentrations induced the greatest expression of the hematopoietic markers CD34, CD43, and CD31 after 6 days of differentiation. CD235 and CD41 expression were not detected throughout differentiation day 6. Increasing concentrations of bFGF induced slight trends of increasing CD31 and KDR expression. Overall, these data indicate that maximal yields of CD31+CD34+CD43+KDR+ cells at day 6 are obtained with mid-range BMP4 concentrations (10-20 ng/ml) during early differentiation, high VEGF concentrations (50-100 ng/ml) throughout differentiation, and mid-range bFGF (5-10 ng/ml) and FLT3 ligand (25 ng/ml) concentrations during late differentiation. Higher concentrations of cytokines displayed no significant increase in yields. We aim to utilize this system progressively to optimize the differentiation of target hematopoietic cells.

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EFFICIENT AND RAPID DOPAMINERGIC DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS BY CO-CULTURE OF EZ SPHERES WITH PA6 STROMAL CELLS

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At present, *in vitro* differentiation of human pluripotent stem cells (e.g., embryonic stem (hES) cells and induced pluripotent stem (hiPS) cells) into subtype neural lineages is inefficient and time-consuming. Recently, various

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approaches including the application of specific chemical compounds inhibiting other cell lineage differentiation, have been reported to improve this process. Here, we report an efficient procedure for *in vitro* differentiation of human pluripotent stem cells to neural precursor cells and then to dopaminergic neurons by combining EZ spheres with PA6 stromal feeders. This novel method provides both fast and efficient generation of neural precursor cells and dopaminergic neurons from human pluripotent stem cells, compared to other currently available methods. We found that EZ spheres derived from hES cells or hiPS cells robustly formed tubular rosettes-like structures as early as 3 days, upon co-culturing with PA6 stromal feeders. Neural precursor cells were generated from rosettes-like structures and were dramatically expanded without losing neuronal differentiation potentials. Subsequently, tyrosine hydroxylase-positive dopaminergic neurons were efficiently and rapidly generated from these expanded neural precursor cells. Taken together, our efficient and rapid method will serve as useful platforms to study neuronal differentiation mechanisms and associated brain disorders using human pluripotent stem cells.

Poster Board Number: 3396

FUNCTIONAL TENDON REGENERATION WITH DIFFERENTIATING HUMAN EMBRYONIC STEM CELLS

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Aim: Activation and repression of lineage-specific genes determine the differentiation fate of embryonic stem (ES) cells. To date, optimal induction of hESC for tendon regeneration has not yet been achieved. Here, we are the first to show that the combination of mechanical stress and transcription factor scleraxis (SCX) is efficient committed the teno-lineage differentiation of hESC. Methods: Human ESCs were first induced to differentiate into mesenchymal stem cells (MSCs). The immuno-phenotype of hESC-derived MSCs (hESC-MSCs) was identified by flow cytometry. Then the hESC-MSCs were transfer with the tendon-lineage specific transcription factor scleraxis. hESC-MSCs formed cell sheet after 14 days culture and engineered tendon were formed *in vitro*. The engineered tendon was subjected to a dynamic mechanical stress of 1HZ for 2h/day. Then the regeneration potential of the engineered tendon tissues was evaluated in both an *in vitro* tissue engineering model and an in-situ rat patellar tendon window repair model. Results: Scleraxis overexpression increased the expression of tendon ECM genes collagen I, XIV.. It was found that SCX can improve the teno-lineage differentiation of hESC-MSC, when working with mechanical stress. SCX has synergistical effect on teno-lineage differentiation but inhibit other mesenchymal lineage differentiation induced by mechanical stress. Also it was demonstrated that combining SCX with mechanical stimulation was able to drive tendon regeneration *in vivo*. Further analysis revealed that enhanced tendon differentiation and regeneration is due to the modulation of BMP signaling by SCX and mechanical stress. Conclusion: Our results demonstrated that functional tendon regeneration can be achieved by hESC-MSC engineered with SCX and mechanical stress. These findings not only contribute to a better understanding of the mechanisms underlying tendon differentiation but also provide new insights in developing novel therapeutic approaches for tendon regeneration using hESC.

Poster Board Number: 3398

CYTOKINE DIRECTED DIFFERENTIATION AND *IN VITRO* SELECTION OF HEPATIC HIPSC AND HESC DERIVATIVES

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Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hIPSCs) hold great promise in regenerative medicine. Due to their merely unlimited self-renewal capacity and their ability to differentiate into derivatives of all three germ layers, these cells might serve as hepatic transplants for metabolic or acute liver diseases. So far, hepatic differentiation efficiencies of hiPSCs and hESCs are well studied, but enrichment of functional active hepatic derivatives for transplantation purposes need further attention. In our study, we aimed for an efficient hepatic differentiation protocol that is applicable for both, hESCs and hiPSCs. We were using a cytokine and small molecule based protocol for direct differentiation of hESC and hiPS cells into hepatic cells. Furthermore, we select the hepatic differentiated cells during differentiation using a hepatic promoter driven G418 resistance. Due to an IRES dependent eGFP reporter expression we were able to track the hepatic differentiated cells and evaluated the most efficient time-frame for G418 selection. The status of the hepatogenic differentiation was determined by qRT-PCR comparing the expression of hepatic markers as AFP, ALB, PCK, SOX17, Cyp1A1 and HNF4 to hepatic cell lines and the loss of pluripotency markers (Oct4, Sox2 and Nanog) to undifferentiated parental cells. Functional analyses of the hepatic phenotype were obtained by measurements of secreted albumin and by analyses of cytochrome P450 type 1A1 activity (EROD). The percentages of differentiated cells were quantified by FACS analyses. Finally, hESC-derived hepatic cells were transplanted into immunodeficient FAH-/- mice. Preliminary data indicate engraftment of transplanted cells into the host liver parenchyma and functional contribution to albumin synthesis. In conclusion, the modified protocol shows that both pluripotent cell sources (hESC and hiPSC) can be differentiated *in vitro* into hepatic cells with similar efficiencies. Further analyses need to reveal the repopulation efficiency of stem cell derived hepatic cells in relation to different pluripotent cell sources and improved differentiation protocols.

Poster Board Number: 3400

A MYOGENIC MEDIUM COMBINED WITH AN ADENOVIRUS EXPRESSING MYOD INDUCE THE MYOGENESIS OF HUMAN EMBRYONIC STEM CELLS.

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Human embryonic stem cells (hESC) have an endless self-renewal capacity and can differentiate into all types of cells found in the body. For this reason, they represent an unlimited source of cells for cell therapy of degenerative diseases, such as Duchenne muscular dystrophy. Due to the considerable amount of myogenic cells required in this kind of cell-based therapy, hESC represents a promising avenue for the elaboration of such treatment. However, at the moment the use of hESC is compromise by the small amount of efficient specific lineage differentiation protocols published. In the case

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of myogenic differentiation, Barberi & al developed a stroma-free induction system to derive engraftable skeletal myoblasts from hESC. However, this technique had a low conversion rate and was time consuming. Since this work, no major break advance has emerged. We developed a new protocol to differentiate hESC into skeletal muscle combining an adenovirus expressing MyoD and a myogenic culture medium (MB1). The fusion potential of these cells was established by the formation of multinucleated myotubes i.e 60% of the nuclei were located in myotubes stained positive for myosin heavy chain. Our results indicate that the combination of MB1 medium and adenovirus is an interesting protocol for hESC differentiation into skeletal muscle cells. The potential of these cells to fuse with muscle fibers *in vivo* is currently under investigation. Our findings are the first step to use hESCs or induced pluripotent stem cells (IPS) as an alternative source of cells for basic applications and for preclinical studies in muscle repair.

Poster Board Number: 3402

RETINOIC ACID ENHANCES SKELETAL MYOGENESIS IN HUMAN EMBRYONIC STEM CELLS BY EXPANDING THE PREMYOGENIC PROGENITOR POPULATION

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Human embryonic stem cells (hESCs) are a potential source of cells for cell therapy of muscle diseases. To date, it has proven difficult to generate skeletal muscle from hESCs in high yields and within a reasonable timeframe. Further, a human ES-derived Pax3/7-positive skeletal muscle progenitor population has not yet been described. Previous studies have shown that Pax3/7-positive progenitor cells can repopulate the satellite cell niche, indicating the importance of this population for therapy. We sought to optimize the differentiation of hESCs into skeletal muscle with a view to identifying distinct stages of myogenesis and shortening the time course. We treated hESCs with retinoic acid (RA) and found that skeletal myogenesis, and the expression of the myogenic regulatory factors (MRFs) Myf5, MyoD and myogenin were enhanced. Furthermore, we found that RA treatment expanded the muscle progenitor pool, which occurred as a distinct Pax3+ve/Meox1+ve population prior to MRF expression. Non-skeletal muscle tissue types, known to be enhanced by RA, were not greatly affected. We have identified a differentiation pathway in hESCs that provides a skeletal muscle progenitor population and a shorter time course of myogenesis. We propose that RA could fit into a directed culture method for deriving skeletal muscle from hESCs.

Poster Board Number: 3404

DISTINCT ENERGY METABOLISM IN HUMAN PLURIPOTENT STEM CELLS AND DIFFERENTIATED CELLS REGULATED BY UCP2

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It has been assumed, based largely on morphologic evidence, that human pluripotent stem cells (hPSCs) contain underdeveloped, bioenergetically inactive mitochondria, while differentiated cells harbor a fused, branching mitochondrial network, with oxidative phosphorylation (OXPHOS) as the main energy source. A role for mitochondria in hPSC bioenergetics therefore remains uncertain. Here we show that hPSCs have functional respiratory complexes that consume O2 at maximal capacity. Despite this, ATP generation in hPSCs is mainly by glycolysis. To help maintain hPSC mitochondrial membrane potential, cell proliferation, and viability, glycolytic ATP is hydrolyzed by the mitochondrial F1F0ATP synthase. Uncoupling protein 2 (UCP2) regulates this unique pattern of hPSC energy metabolism while suppressing reactive oxygen species. Upon differentiation, UCP2 is repressed and cells shift from glycolysis and fatty acid oxidation to glucose oxidation dependency. Ectopic UCP2 expression during differentiation blocks this metabolic transition, and it blocks induction of differentiation genes and proper embryoid body formation. Combined, our data show that hPSC have distinct bioenergetics regulated by UCP2 and repression of UCP2 is required for metabolic reprogramming and proper differentiation. This is to our knowledge the first report on a energy metabolism-related molecule regulating differentiation in hPSCs.

Poster Board Number: 3406

GENERATION AND CHARACTERIZATION OF INDUCED PLURIPOTENT STEM CELLS FROM HUMAN SARCOMA CELL LINES

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Induced pluripotent stem cells (iPSCs) are defined as somatic cells that have been reprogrammed (using either the four transcriptional factors Oct4, Sox2, Klf4 and c-Myc or Oct4, Sox2, Nanog and Lin28) to exhibit embryonic stem cell-like pluripotent differentiation properties. The generation of iPSCs was a great achievement for the field of cell transplantation and tissue engineering. More recently multiple cancer cell lines (e.g, leukemia cells, melanoma cells & gastric cancer cell) have been successfully reprogrammed to the iPSC-state, at least as defined by (1) expression of genes specific to undifferentiated embryonic stem cells; and (2) pluripotency as defined by embryoid body formation *in vitro* and early markers of commitment to various differentiation lineages. However, the question that remains unanswered is: can the cancer derived iPSCs complete maturation along a given lineage, be it the lineage from which it was derived (i.e., originally prematurely blocked) or an alternative lineage? If the former were demonstrated it would in essence answer the question of whether global changes in chromatin architecture or epigenetics can supersede the specific genetic changes (i.e., mutation) in DNA that resulted in the lineage specific differentiation block and subsequent tumorigenesis. To attempt to answer these questions we generated iPSC-cancer cells from sarcoma cell lines representing undifferentiated sarcomas, osteosarcoma cells, liposarcoma cells, and sarcomas of unknown lineage. While the original sarcoma cell lines could not be differentiated into multiple mature connective tissue cells, their iPSC-counterparts readily differentiated into multiple mature connective tissue lineages. Furthermore, assessment of proliferation indicated that acquisition of the mature phenotype was concurrent with cessation of proliferation; and thus abrogation of tumorigenicity. We are currently(1) performing gene expression, microRNA, and global DNA promoter methylation profiling in order to assess the mechanism by which iPSC-based reprogramming of cancer cells overrides the inherent genetic mutation in these cells and restores the capacity for terminal differentiation and (2) extending our work to carcinomas.

Poster Board Number: 3408

DIRECTING HUMAN EMBRYONIC STEM CELL DIFFERENTIATION BY NON-VIRAL DELIVERY OF SIRNA IN 3D CULTURE

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Human embryonic stem cells (hESCs) hold great potential as a resource for regenerative medicine. Before achieving therapeutic relevancy, methods must be developed to control stem cell differentiation. It is clear that stem cells can respond to genetic signals, such as those imparted by nucleic acids,

to promote lineage-specific differentiation. However, the major barrier preventing the use of nucleic acids from influencing differentiation is the lack of safe and efficient delivery methods. The majority of current delivery methods utilize modified viruses. While viruses are an effective means of transferring nucleic acids to stem cells, they have limited loading capacity and questionable safety. Here we have developed an efficient system for delivering siRNA to hESCs in a 3D cultured matrix. We show that efficient siRNA delivery can be used to coordinate hESC differentiation. Using siRNA to silence either the KDR or the androgen receptor gene, we achieve concurrent down regulation in genes representative of the endoderm germ layer and significant upregulation of genes representative of the mesoderm germ layer. We also identify that integrins play a significant role in the siRNA delivery and activity. This is the first demonstration that siRNA can direct stem cell differentiation by blocking one of the germ layers.

Poster Board Number: 3410

COMPARISON OF VENTRAL PROSENCEPHALIC PROGENITORS GENERATED FROM HUMAN AND MOUSE EMBRYONIC STEM CELLS THAT GIVE RISE TO MATURE CORTICAL NEURONS

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The developing prosencephalon gives rise to cortical, limbic, and hypothalamic structures in the brain, which in the human are the regions that underlie complex cognitive processes, including movement, memory, and executive decision-making. While some mechanisms to generate this vast diversity of cell types are conserved between mammalian species, several processes have already been described in humans to be evolutionarily divergent. By comparing the mouse and human prosencephalon during embryonic development, we have identified the molecular signatures that are common and unique to humans. In this study, we seek to elucidate the signaling pathways involved in patterning the ventral prosencephalon through the use of human and mouse embryonic stem cells (ESCs). By directing their patterning at the pluripotent stage, we can direct these cells towards various diencephalic and telencephalic progenitors within two to three weeks through a novel rapid induction protocol. Using ESCs that express GFP regulated by the NKX2.1 promoter, we can isolate ventral prosencephalic progeny through fluorescent activated cell sorting (FACS). When FACS-sorted and placed into a cortical environment, either *in vivo* or *in vitro*, these GFP-expressing progenitors give rise to GABA-expressing neurons that receive inputs and can fire action potentials reminiscent of cortical interneurons. Therefore, this work demonstrates the use of human ESCs to model human development. By generating a plethora of specified human ventral prosencephalic progenitors, this work can be applied to the study of distinct and novel neural populations.

Poster Board Number: 3412

GENESIS OF RETINAL PIGMENT EPITHELIUM CELLS DURING MONOLAYER DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS

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Age-related macular degeneration (AMD) is the most common cause of irreversible visual loss in the elderly in the Western world. It is a devastating disease of the retina that can lead to central blindness. It affects the retinal pigment epithelium (RPE) - the layer of cells that surrounds and nourishes the neurosensory retina. When RPE cells do not function properly, photoreceptor cells begin to die and vision is lost. Currently, there are few if any available option to treat or replace diseased RPE. Cell-based transplantation strategies offer the promise of being able to restore RPE cells, thus potentially limiting vision loss. Therefore, establishing an abundant and quality source of donor cells is of utmost importance. Human pluripotent stem cells (hPSCs) may prove suitable for this purpose: significant advances have recently been made in inducing the differentiation of hPSCs toward an RPE-like cell fate. Nevertheless, the length and efficiency of these methods are still not optimal. Current RPE differentiation protocols are based on embryoid body or cell monolayer-based differentiation approaches. The relative simplicity of the latter approach makes it a preferred alternative for high throughput screening campaigns aimed at finding small molecules that could improve RPE differentiation efficiency. However, RPE generation from hPSC is a lengthy process (up to several months). Therefore, it would be most useful to first gain a precise understanding of the time frame and efficiency of the different steps involved in RPE differentiation, in order to adapt high throughput screening strategies. Since very little is known regarding the different steps of RPE genesis during hPSC monolayer differentiation, we decided to start studying these, with a view to establishing a small molecule screen for compounds that promote RPE differentiation and survival. Following a simple and scalable monolayer differentiation protocol with serum-free and feeder-free conditions, we were able to obtain RPE-like cells from human embryonic stem cells, as well as from induced pluripotent stem cells. hPSC derived RPE cells formed a pigmented monolayer, and expression of specific markers such as Mitf, Otx2, CRALBP and Best1 was confirmed at the mRNA and protein levels. On the functional level, pigmented RPE sheets were able to phagocytose pHrodo labeled bioparticles. Since pigmented cell clusters started to appear after 25-30 days of *in vitro* differentiation, we next sought to follow the expression of key transcription factors involved in RPE differentiation during this time window. Preliminary results indicate that after a surge in the number of RPE precursor cells (Mitf+/Otx2+) at 5 days of differentiation, significant cell loss is observed 5 days later. Therefore, the first 10 days of differentiation might constitute a window of opportunity to screen for small molecules able to promote survival and proliferation of RPE precursors.

Poster Board Number: 3414

LINKING DIFFERENTIATION AND CELL CYCLE WITHDRAWAL IN HUMAN EMBRYONIC STEM CELLS

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During the earliest stages of development nearly all cells in an embryo undergo continuous cell division to increase the total cellular mass of the embryo. As development progresses cell specialization occurs and an increasing number of cells begin to withdraw from the cell cycle and differentiate into multiple cell types. One of the unanswered questions in this process is whether differentiation initiates cell cycle withdrawal or whether cell cycle withdrawal enables differentiation. The differentiation of human embryonic stem cells is an excellent system with which to model this process. We and others have shown that nearly all pluripotent hESC remain in the cell cycle and that their cell cycle consists of very short G1 phases and long S phases. However, as these cells begin to differentiate, an increasing number of cells withdraw from the cell cycle and the length of S phase decreases with a corresponding increase in the length of the G1 phase. Concomitantly with lengthening of the G1 phase, negative regulators of the cell cycle such as p21/WAF1 and p27/KIP1 increase. We hypothesize that cell cycle withdrawal is necessary for a cell to undergo differentiation and are using this model system to investigate the mechanisms that link the differentiation of cells to their withdrawal from the cell cycle.

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Poster Board Number: 3416

CANONICAL WNT SIGNALING INHIBITS MEGAKARYOCYTE-ERYTHROID PROGENITOR FORMATION FROM HUMAN EMBRYONIC STEM CELLS

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The Wnt gene family consists of structurally related genes encoding secreted signaling molecules that have been implicated in many developmental processes, including regulation of cell fate and patterning during embryogenesis. We have previously found that Wnt signaling enhances primitive erythrocyte development and its inhibition results in a loss of primitive erythrocyte development when given to mouse ES cell-derived mesoderm. Here, we examine the effect of Wnt signaling on the formation of early hematopoietic progenitors derived from human ES cells. The first hematopoietic cells co-express CD41 and CD235, typical markers of megakaryocytes and erythrocytes, respectively. We find that canonical Wnt signaling is inhibitory both to the development of this hematopoietic population from mesoderm and to its maintenance. Specifically, treatment of mesodermal cells with Wnt3a led to an almost complete loss in the development of colony-forming cells while inhibition of canonical Wnt signaling with DKK1 led to a 4-fold increase in erythroid colony formation. Culturing CD41+CD235+ cells with Wnt3a led to a 10-fold decrease in the number of CD41+CD42+ megakaryocytes and CD235+CD41- erythroid cells. Addition of the non-canonical Wnt5a has a similar effect as DKK1 treatment in that it increased the generation of CD41+CD235+ progenitors. In addition, we demonstrate that this effect is specific to primitive erythrocyte-megakaryocyte progenitors, as myeloid development is less affected by Wnt3a treatment. This finding is in stark contrast to the role of Wnt signaling during mouse ES cell differentiation. These studies demonstrate the utility of the ES cell system in the study of cell fate decisions and highlight the importance of working with human ES cells as there are some species- specific differences in development that will need to be addressed before applying findings from the mouse model system.

Poster Board Number: 3418

DEFINING TRANSCRIPTIONAL LANDMARKS OF EARLY HUMAN DEVELOPMENT WITH HUMAN PLURIPOTENT STEM CELLS

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Human pluripotent stem cells (hPSCs) have the capacity to differentiate to all cell types of the human body making them very attractive for regenerative medicine purposes. It is not known, however, if *in vitro* differentiation of hPSCs mirrors *in vivo* development, nor if the differentiated progeny of hPSCs are truly analogous to their tissue-derived counterpart. To answer these questions, we have differentiated two sources of hPSCs, human embryonic stem cells and human induced pluripotent stem cells, to cell types representing all three germ layers: a neural progenitor cell (ectoderm), a hepatocyte (endoderm), and fibroblasts (mesoderm). We performed large scale transcriptome profiling comparing PSC progeny to their tissue-derived counterparts and identified a significant list of genes that is differentially expressed between the PSC derivatives and their natural counterparts regardless of germ layer produced. The observed differences include many genes expressed in the early embryo that fail to turn off during *in vitro* differentiation. These data and evidence from expression patterns in early human fetal tissue (3-16 weeks of development) suggests that the differentiated progeny of human PSCs are more reflective of very early human development (<6 weeks gestation) rather than later time points. These findings validate the idea that human pluripotent stem cells can serve as useful models of very early development, but also underscore the need to consider the maturity of hPSC derivatives for regenerative medicine purposes.

Poster Board Number: 3420

DERIVATION OF ASTROCYTES FROM HUMAN EMBRYONIC STEM CELLS FOR SPINAL CORD INJURY THERAPY

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Every year about 300,000 patients in the U.S. suffer traumatic injury of the brain or spinal cord requiring hospitalization, of which 250,000 survive. Due to the limited regenerative capability of adult central nervous system (CNS), these patients are facing the debilitating consequence of their injuries. Taking advantage of the neuroregeneration promoting properties of glial cells, we have developed a cell therapy for SCI using a specific type of astrocyte. Previously we have shown that transplantation of BMP-induced astrocytes (GDAsBMP) derived from either rat or human embryonic glial-restricted precursor (GRPs) cells promotes extensive neuronal survival, axonal regeneration, and functional recovery in a rat spinal cord lesion model. With the recent advances in human embryonic stem cell (hESC) technology, we hypothesize that hESCs can be used as a source for generating astrocyte populations with therapeutic potential. Using step-wise differentiation through an intermediate neural progenitor stage, we have derived distinct types of human astrocytes from hESCs. Our data indicate that these hESC-derived astrocytes recapitulate characteristics of rodent precursor-derived astrocytes with respect to cell morphology and expression of glial specific genes. We have also developed a high-throughput *in vitro* neuronal survival and axonal outgrowth assay using the Cyntellect Celigo adherent cell cytometer. Our Celigo results show that astrocytes derived from hESC by BMP induction selectively support the survival and axonal growth of embryonic cortical and dorsal root ganglion neurons. These results set the stage for testing the therapeutic potential of hESC-derived astrocytes for spinal cord injury regeneration *in vivo*.

Poster Board Number: 3422

A REPORTER HUMAN EMBRYONIC STEM CELL LINE THAT FACILITATES IDENTIFICATION OF THYMIC EPITHELIAL CELLS DURING DIFFERENTIATION

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Thymic epithelial cells (TECs) form the main constituent of the thymus microenvironment and play an essential role in thymocyte development and T-cell repertoire selection. The ability to produce a replacement source of TECs from human embryonic stem cells (hESCs) would significantly enhance research efforts into the mechanisms that govern immunity, and may enable the restoration of thymus function in aged or diseased states. Furthermore, hESC-derived TECs could theoretically be combined with host-derived haematopoietic stem cells (HSCs) to create a chimeric thymus, which in turn might be used to establish antigen-specific tolerance induction *in vivo*. In order to facilitate analysis of directed thymic differentiation, we have developed a hESC (FOXN1GFP/w) line in which the green fluorescent protein (GFP) gene has been inserted into the locus encoding the forkhead-box transcription factor, FOXN1. Expressed in all epithelial cells of the early

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thymic rudiment, FOXN1 is an essential gene for TEC differentiation into mature cortical and medullary epithelial cells. Correct gene targeting was confirmed using a PCR-based approach in conjunction with southern blot hybridisation. FOXN1GFP/w lines are karyotypically normal, retain expression of pluripotency markers and form multi-lineage teratomas in immunodeficient mice. We have used our FOXN1GFP/w hESCs in conjunction with a serum-free spin embryoid body (EB) differentiation system to develop a robust and reproducible protocol for the generation of FOXN1+ endodermal progenitors. Analysis of this population confirms that FOXN1 expression is confined to the GFP+ fraction and that these cells express a suite of markers consistent with early foregut endoderm. Current experiments are focused on further characterising purified differentiated FOXN1+ cells via transcriptional profiling, functional assays and transplantation experiments.

Poster Board Number: 3424

DEATH RECEPTORS EXPRESSION AND RESISTANCE TO TRAIL-INDUCED APOPTOSIS IN HUMAN EMBRYONIC STEM CELLS AND THEIR DIFERENTIATED DERIVATIVES

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Implantation of *in vitro* differentiated human embryonic stem cells (hESC) derivatives constitutes promising therapy of multiple serious diseases. Survival of grafted cells is influenced by several factors, expression of death receptors and their pathway components being an important one. In living organism expression of death receptors functions to eliminate transformed cells. Elevated expression of TRAIL receptors DR4 and DR5 sensitizes these cells to TRAIL-induced apoptosis engaging caspase 8. Expression of death receptors, constituency of downstream signalling and its functionality in undifferentiated hESCs and their differentiated derivatives has not been explored yet. We determined expression of death receptors during the course of differentiation of hESCs into neural precursor cells line (NPC), expression of caspase 8 and response to TRAIL. Quantities of death receptors (DR4, DR5, DcR1, DcR2, TNFR1, Fas) were determined by flow cytometry. We found that expression profile of death receptors changed with differentiation. Whereas quantity of DR4 during differentiation decreases, DR5 and Fas increases. TRAIL decoy receptors DcR1, DcR2 are not significantly expressed in none of investigated cell types. Both undifferentiated hESCs and NPCs express caspase 8, essential for execution of TRAIL induced apoptosis. Interestingly, treatment with soluble recombinant TRAIL (0-1000ng/mL, 24h) did not induce apoptosis either in hESCs or NPCs, but cells were sensitized by chemical inhibition of proteasome (1μM MG132, 24h). Our work describes presence of selected components of death receptor apoptotic pathway in undifferentiated human embyonic stem cells and its differentiating derivatives - neural precursor cells. However, presence of these components themselves is not sufficient to mediate apoptosis upon TRAIL treatment. Our current research effort focuses on detailed understanding of mechanism of resistance to TRAIL induced apoptosis.

Poster Board Number: 3426

MODULAR CHEMICALLY DEFINED CULTURE SURFACES SUPPORT HUMAN EMBRYONIC STEM CELL-DERIVED PROGENITOR CELLS FROM EACH OF THE PRIMARY GERM LAYERS.

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Human embryonic stem cells (hESCs) hold promise to treat diseases such as Parkinson's and type I diabetes, which are caused by the destruction of specific cell types. In order to achieve the promise these pluripotent cells hold, precise control of hESC fate and differentiation must be established. Here we have employed a modular and defined set of peptide surfaces to support undifferentiated hESCs and hESC-derived progenitor cells from each germ layer. Significant research is dedicated to creating completely defined culture conditions, which are important to reduce experimental variability and to prevent exposure of cells to pathogens or xenobiotics. Researchers have access to commercial chemically defined media, but developing a completely defined matrix has proven more difficult. Recently, we discovered that surfaces displaying the peptide GKKQRFRHRNRKG derived from the heparin-binding domain of vitronectin are capable of supporting long term undifferentiated hESC culture. The surfaces consist of biotinylated peptides bound to streptavidin-coated polystyrene. Moreover the surfaces are modular in that by mixing different peptide conjugates together, they can display multiple peptides or peptides in various densities. Surfaces displaying GKKQRFRHRNRKG alone support hESCs in the presence of Y-27632 ROCK inhibitor while surfaces presenting an equal mixture of GKKQRFRHRNRKG and a cyclic RGD peptide require ROCK inhibition only during initial cell plating. Further, hESCs cultured long term under these defined conditions retain the ability to respond to directed differentiation protocols to ectoderm, endoderm, and mesoderm. Because most adherent directed differentiation protocols rely on undefined matrix components such as Matrigel or gelatin, this aspect of the project instigated research into designing tailored synthetic niches for hESC-derived progenitor cells from each germ layer. We have shown that hESCs grown on GKKQRFRHRNRKG in the presence of Y-27632 can differentiate into PAX6+ ectoderm when treated with Noggin and SB-431542 for 6 days, SOX17+ definitive endoderm when treated with Activin A for 6 days, and FOXF1+ mesoderm when treated with Activin A for 1 day and BMP4 for 5 days. All of the differentiation conditions involved the defined matrix and serum-free media. However, unlike undifferentiated hESCs, the combination of GKKQRFRHRNRKG and cyclic RGD does not support differentiation toward the ectoderm in the absence of ROCK inhibitor. In order to address this issue, we have compared the expression of cell adhesion receptors and extracellular matrix (ECM) proteins of hESC-derived PAX6+ OCT4- neural precursor cells to undifferentiated hESCs. We plan to design surfaces that display ligands to differentially expressed cell adhesion receptors or that mimic bioactive regions of differentially expressed ECM proteins. In conclusion, we show that surfaces displaying the peptide GKKQRFRHRNRKG can serve as a defined matrix for hESC directed differentiation protocols to each germ layer when ROCK is inhibited. Additionally we are developing surfaces that present ligands to differentially expressed cell adhesion receptors in order to improve directed differentiation protocols in terms of cell viability, yield of the desired cell type, and independence from soluble molecules such as ROCK inhibitor. These surfaces offer modular, simple and accessible defined culture matrices for hESCs and hESC-derived progenitor cells from each germ layer.

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Poster Board Number: 3428

DIFFERENTIATION OF HESCS INTO CARDIAC LINEAGE BY MODULATION OF BOTH ALK/SMAD AND BFGF SIGNAL PATHWAY

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The TGFβ/BMP-activated Smad signaling pathway plays a complicated role in the maintenance of human embryonic stem cell (hESC) pluripotency and in the cell fate decision of hESCs. To better understand the specific role of the TGFβ/BMP signaling pathway in hESC function, we examined the effects of a pharmacological ALK4/5/7 inhibitor, which blocks type I receptor-mediated Smad signaling, on *in vitro* hESC differentiation under various culture conditions with or without bFGF stimulation. Here, we report that sustained inhibition of the TGFβ type I receptor (also termed activin receptor-like kinase or ALK) using a chemical inhibitor selective for ALK4/5/7 (ALKi) leads to the cardiac differentiation of hESCs under feeder-free and serum-free conditions. Treatment with ALKi reduced Smad2/3 phosphorylation and increased Smad1/5/8 phosphorylation in hESCs, suggesting a requirement for active Smad1/5/8 signaling for cardiac induction in these cells when ALK/Smad2/3 is inhibited. Importantly, active bFGF signaling was also required for ALKi-mediated cardiac differentiation of monolayer-cultured hESCs. The FGFR inhibitor SU5402 blocked ALKi-mediated cardiac induction in hESCs, while BMP4 enhanced the ALKi-induced increase in phospho-Smad1/5/8 levels but failed to induce the cardiac differentiation of hESCs, and instead promoted trophoblastic differentiation. We also confirmed that ALKi potentially enhanced the cardiac differentiation of hEBs, as determined by expression of cardiac specific markers, increased beating areas, and action potential recorded from beating areas. Our results demonstrate that ALKi could serve as a potent cardiogenic inducer of hESCs, and coordinated Smad-dependent signaling and FGF-dependent signaling play an important role in the differentiation of hESCs into the cardiac lineage. Acknowledgements: The study was supported by grants of the NRF grant [2010-0020272(3)] and the KRCF/KRIBB research initiative program.

Poster Board Number: 3430

IDENTIFICATION OF A SYNTHETIC NANOFIBRILLAR MATRIX THAT PROMOTES HEPATIC DIFFERENTIATION OF MOUSE AND HUMAN ES CELLS AND IPS CELLS *IN VITRO*

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In drug discovery, many researchers often use primary cultures of hepatocytes for toxicity tests. However, primary hepatocytes are short-lived and cannot be maintained in long term cultures. In addition, there are considerable donor dependent variations. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst. Lines of evidence have shown that ES cells recapitulate normal developmental processes, and suggest that ES cells provide an attractive source for routine access to large numbers of cells that enable the development of new drug-screening strategies. We previously reported that culturing ES cells on a mesonephric cell line, M15, with the addition of specific growth factors resulted in a selective induction of hepatic differentiation of ES cells at a high efficiency. Here, we established a novel hepatic differentiation method using synthetic nanofiber (NF) as a cell culture scaffold. First, we performed endoderm differentiation of mouse ES (mES) cells using NF based methods in comparison with several substrata, such as gelatin, fibronectin, collagen I, and MHS gel. Similar to collagen I and MHS gel, ES cells cultured on NF methods gave rise to Sox 17 positive endodermal cells efficiently. Using these three substrata, we cultured further for 20 days until ES cell became mature. Time dependent Albumin expressions and secretions were analyzed by RT-PCR and ELISA, respectively. On differentiation day 24, functional assays for hepatocyte were also carried out. In these assays, PAS- assaying glycogen storage, and indocyanin green (ICG) test for hepatocyte detoxification function, and cytochrome P450 activities and their induction responding to inducers were analyzed. All these assays revealed that ES cells cultured on NF were potentiated for hepatic differentiation. Moreover, differentiated ES cell-derived hepatocytes uptook ICG and were maintained for more 100 days on NF. These results indicate that NF is a suitable scaffold that not only promotes ES differentiation into hepatocytes, but also maintains the differentiated functions of the hepatocytes. Finally, we checked whether NF method was applicable to human ES cells and human induced pluripotent stem (iPS) cells. As a result, NF is similarly effective in supporting the hepatic differentiation of hES cells as compared to M15 cells. As this NF method is a serum-free and feeder-free system, it would be useful for Xeno-free culture of human ES/iPS cells. This novel procedure will be useful for the elucidation of molecular mechanisms that underlie lineage decision, and could represent a key milestone for a surrogate cell source not only for regenerative medicine but also for pharmaceutical studies such as toxicology.

Poster Board Number: 3432

3D ELECTROSPUN NANOFIBROUS SCAFFOLDS ENHANCE MOUSE CARDIOVASCULAR PROGENITOR CELLS *IN VITRO*

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Tissue engineered structures are fast becoming a viable solution for not only the treatment of heart disease by replacing diseased cardiovascular tissue, but also as a means to further understand the complex nature of cardiovascular tissue development. We have previously seen that common extracellular matrix (ECM) proteins have varying effects on the terminal differentiation of murine embryonic stem (ES) cells. It has also been previously thought that three-dimensional (3D) cell culture environments more closely mimic the *in vivo* native environment. In an effort to elucidate the mechanism through which the complex ECM 3D microenvironment enhances cardiovascular differentiation of ES cells, we have examined the partially differentiated cardiac progenitor cells (CPCs) in different 2D and 3D conditions. Co-axial bicomponent electrospun scaffolds were created from a core of 5% polyurethane (PU) and a sheath of a hybrid mix of 10% polycaprolactone- 10%gelatin. Briefly, the solutions were loaded into a double syringe system with a 25G blunt edge needle for the core solution and an 18G needle for the sheath solution. An applied voltage of 30kV was used with a flow rate of 70μL/min and a grounded collection plate 16.5cm away. The following protein coatings were applied to a standard 24-well culture dish as well as 3D electrospun scaffolds: gelatin, collagen IV, laminin, fibronectin, and vitronectin. mES cells were expanded in a feeder free cell culture conditions using ESGRO medium. Cells were kept in an undifferentiated state until ready for seeding. Each 2D or 3D condition was seeded with 500,000/cm2 undifferentiated mES cells. Once seeded, the cells were maintained in differentiation media, α-MEM media supplemented with 10% FBS. Initial cytotoxicity assays confirm the biocompatible nature of the protein coatings and electrospun nanofibrous scaffolds. These studies have also confirmed the prolonged presence of the protein coatings for the duration of the current study, up to 9 days of culture. FACS analysis shows a gradual decrease in CPC population (Flk-1+) when exposed to collagen IV in 2D vs. 3D conditions. However,

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collagen IV in 2D conditions do exhibit high levels of Flk-1+. Laminin and vitronectin 3D conditions support higher number of Flk-1+ as compared to their 2D counterparts. As previously reported CPCs as identified by Flk-1 have shown greater differentiation potential when exposed to various ECM proteins. Additionally, a 3D microenvironment further enhances this differentiation potential. In order to determine the mechanism by which a 3D microenvironment enhances a cellular response, we have combined both the 3D microenvironment of the electrospun nanofibrous scaffolds with various ECM proteins we have identified as influential. We have seen that the 3D microenvironment has the ability to enhance the number of CPCs present after at least 4 days in differentiation conditions. Further study will be required to determine the appropriate *in vitro* microenvironment to fully recapitulate the cardiovascular stem cell niche present in the heart and understand the specific cellular mechanisms of how these ECM proteins are able to enhance CPC populations.

Poster Board Number: 3434

REGULATION OF CARDIOMYOGENESIS IN MOUSE EMBRYONIC STEM CELLS BY GLIOMA ASSOCIATED FACTOR 2

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Embryonic stem (ES) cells have the ability to differentiate into a variety of tissue types such as cardiac myocytes and, therefore, offer great therapeutic potential. Identifying the role of transcription factors important for cardiomyogenesis could be instrumental in developing heart muscle stem cell therapies. Our lab has previously shown that the overexpression of Glioma associated factor 2 (Gli2) induces cardiomyogenesis in P19 embryonal carcinoma (EC) cells, a well-established model of ES cells. Alternatively, disrupting Gli2 expression in P19 EC cells delayed cardiomyogenesis. We hypothesize that Gli2 has a similar role in the transcriptional regulation of cardiomyogenesis in murine ES cells. Murine ES cells, stably overexpressing Gli2, were grown *in vitro* under optimal cardiomyogenesis committing conditions. Quantitative polymerase chain reaction (qPCR) indicated that Nkx2.5 and Tbx5, markers of cardiomyogenesis; MEF2C, a gene expressed during cardiomyogenesis; and MHC6, a cardiac muscle specific myosin heavy chain, were upregulated in cells containing stably overexpressed Gli2, while mesoderm markers, Mesp1 and Brachyury T, were unaffected. Our data suggests that the overexpression of Gli2 in murine ES cells has a positive effect on cardiomyogenesis, in accordance with previous studies performed in P19 EC cells.

Poster Board Number: 3436

THE EFFECT OF BETA-BOSWELLIC ACID ON DOPAMINERGIC PROGRAMMING OF MOUSE EMBRYONIC STEM CELLS

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Parkinson disease (PD) is a neurodegenerative disorders in which dopaminergic (DAergic) neurons in substantia negra region are degenerated. Cell replacement therapy can surmount the obstacles related to current PD chemotherapy. Here, for the first time the effect of Beta-Boswellic acid (BBA) as major component of Boswellia serrate gum on DAergic differentiation of mouse embryonic stem (mES) cells has been investigated. We already demonstrated the enhancement effect of BBA on neurites growth of neurons isolated from hippocampus. To end that, we used two R1 and CGR8 cell lines, feeder dependent and independent mES, respectively. The cells were subjected to EB formation in medium contains 3% serum replacement for one week. Then the ES-differentiating cells were embedded by Matrigel as a natural extra cellular matrix and plated on Matrigel coated plates. Several concentration of BBA (10-50nm) used in the step three of gold standard protocol for DAergic differentiation. Then the efficiency was analyzed by Real-time RT-PCR, Immunocytochemistry for expression of neuroectermal, mesencephalic or DAergic neuron-related markers expression such as Nestin, Pax2, Pax5, Wnt, En, Nurr1 and TH. As a positive control SY5Y, a neuroblastoma dopamine-synthesizing cell line was used for our analyses. The synthesis and secretion of dopamine will be analyzed by reverse HPLC in supernatant of differentiated cells. The result showed that embedding and plating of EBs regardless to BBA concentrations on Matrigel, increase the survivals of the differentiated cells, and expression of transcripts related to mesencephalic and DAergic neurons. The results showed the high DAergic differentiation is achieved in medium contains 30 nM BBA; however the expression of mesencephalic markers such as Pax2 and Pax5 in 10nM BBA was more than 30nM. In conclusion, BBA can promote DAergic differentiation of mES cells that this effect can be synergized by presence of Matrigel. The mechanism of DAergic programme promotion by BBA in ES is under investigation. The result of this study may have impact on future cell therapy of PD by ES or induced pluripotent stem cells (iPS).

Poster Board Number: 3438

GENERATING LIMB-INNERVATING MOTOR NEURONS THROUGH THE MISEXPRESSION OF FOXP1 IN MOUSE EMBRYONIC STEM CELLS

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With the ability to generate spinal motor neurons (MNs) from stem cells in culture, great hope has been placed on using stem cell-derived MNs to treat neurodegenerative disease by both replacing damaged neurons and creating *in vitro* models to study disease pathogenesis. However, the methods currently used to generate MNs from embryonic stem cells (ESCs) typically produce a limited subset of the MN subtypes that exist *in vivo*. We hypothesized that the forced expression of different MN fate determinants could efficiently direct the formation of specific MN classes. Specifically, we tested whether misexpression of the transcription factor Foxp1 in mouse ESCs was sufficient to generate limb and sympathetic nervous system-innervating MN populations. Transgenic mice expressing Foxp1 in all spinal MNs show increased numbers of limb-innervating MNs and reduced MN axonal projections to axial muscles, specifically the rhomboideus muscle. We derived mouse ESC lines that express Foxp1 under the MN-specific promoter Hb9, and upon differentiation of these lines we are able to generate MNs (with ≤30% efficiency), of which 80% express Foxp1. Of these Foxp1+ MNs, 58% and 42% express molecular profiles characteristic of dorsal (Lhx1+) and ventral (Isl1+) limb-innervating MNs, respectively. The Foxp1+ MNs express HoxA5 and HoxC6, which indicates a rostral forelimb identity. In addition, the Foxp1+ MNs also express the EphA4 receptor, which is highly expressed by dorsal limb-innervating axons *in vivo*. Upon transplantation of the Foxp1+ MNs into developing chick embryos, they project axons to both the dorsal and ventral limb. Lastly, the Foxp1+ MNs are physiologically active and form synapses when cultured with muscle. These results provide important insights into whether different types of MNs can be generated in culture simply by altering expression of specific MN fate determinants.

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ESTABLISHMENT OF AN EFFICIENT NEURAL CREST CELL INDUCTION SYSTEM FROM MOUSE EMBRYONIC STEM CELLS IN GROWTH FACTOR DEFINED SERUM-FREE MEDIUM

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The neural crest is a stem cell-like population that arises at the boundary between the neural and non-neural ectoderm. Neural crest (NC) cells give rise to a vast range of cells. However, the molecular mechanisms of NC induction during early development remain poorly understood. Embryonic stem (ES) cells could be used for an *in vitro* model of mammalian development. Although conventional differentiation methods using ES cells usually involve forming embryoid bodies and culturing in medium that contains undefined components. Therefore, these methods have hardly enabled us to follow up the process of cell status change during differentiation. We previously developed a growth factor defined serum-free medium for culturing mouse ES (mES) cells without feeders. Under this defined culture condition, the effects of additive factors on ES cells are expected to be clearly assessed. In this report, we establish a NC induction system from mES cells in a growth factor defined serum-free monolayer culture condition. We assessed the effects of extracellular matrix components and found that laminin matrix is effective for the differentiation of mES cells into neural cell lineages. We found that FGF-2 upregulated expression of primitive ectodermal genes and subsequently elevated expression of neuroectodermal genes. We also found that BMP-4 upregulated expression of NC genes in FGF-2 treated cells. These results indicate that adding BMP-4 together with FGF-2 promotes mES cells differentiation into the NC through lineage-specific defferentiation. Our data also showed that these FGF-2/BMP-4 treated cells have the potential to differentiate into NC derivatives such as peripheral neurons, Schwann cells, smooth muscle cells, adipocytes, chondrocytes, and osteocytes. Furthermore, we have established a cell line designated as P0-6 that is derived from the blastocysts of P0-Cre/Floxed-EGFP mice expressing EGFP in an NC-lineage-specific manner. P0-6 cells cultured using this culture system expressed EGFP. Taken together, this growth factor defined serum-free NC induction system could be useful tool for studying the mechanism of by which cells differentiate into the NC and for developing stem cell therapies.

Poster Board Number: 3442

IMMUNOMAGNETIC ENRICHMENT OF MOUSE EMBRYONIC STEM CELL DERIVED NEURONAL PRECURSORS PRIOR GRAFTING INCREASES THE NUMBER OF *IN VIVO* DIFFERENTIATED NEURONS

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Several protocols for differentiation of mouse pluripotent stem cells (mESC, iPSC) into specific neural subpopulations were developed by use of specific signaling factors. Nevertheless, such protocols are not able yet to synchronize the generation and development of specific neural subpopulations, except for nestin+ neural progenitors, arising to cellular heterogeneity that hampers experimental and clinical improvement in the use of pluripotent stem cells. In order to generate a clearly defined population of neuronal precursor cells, we characterized the expression of cell surface markers of actin-eGFP mESC differentiated into neuronal subpopulations. We observed the presence of PSA-NCAM positive neuronal precursors and developed a method for isolation of mESC derived PSA-NCAM positive cells by immunomagnetic sorting. PSA-NCAM, as neural surface marker, plays a role in neural plasticity from embryonic to adult nervous system (axonal growth, cell migration, synaptic plasticity, and embryonic and adult neurogenesis). For functional characterization, we enriched the *in vitro* derived PSA-NCAM+ cells with magnetic beads and compared their *in vivo* behavior to unsorted mESC derived nestin+ cells by injecting both cell populations stereotactically into the forebrain of adult mice. Analysis for several neuronal and glia markers (PSA-NCAM, TUJ1, NeuN, MAP2, GAFP, Olig2, GLAST) after 10 or 40 days post graft, showed a distinct differentiation pattern of the two grafted populations. PSA-NCAM+ actin-eGFP derived neuronal precursors retained their ability of migration and the majority differentiated in NeuN (marker for mature neurons) positive cells. On the other hand, actin-eGFP mESC derived nestin+ cells differentiated only rarely into NeuN positive cells but gave rise to a mixed population of cells expressing markers for neuronal precursors and young neurons such as PSA-NCAM and TUJ1, respectively. Moreover, a higher number of actin-eGFP PSA-NCAM expressing cells migrated along the rostral migratory stream (RMS) in those animals where the PSA-NCAM+ actin-eGFP enriched population was grafted. Taken together, these results show that a straight forward *in vitro* enrichment of functional ES cell derived neuronal precursor cells can be used to streamline the *in vivo* differentiation towards neurons and increase the number of target cells after grafting.

Poster Board Number: 3444

THYROID HORMONE PROMOTES NEURONAL DIFFERENTIATION AND INHIBITS THE PROLIFERATION AND MAITENANCE OF MOUSE EMBRYONIC NEURAL STEM CELLS THROUGH THYRIOD HORMONE RECEPTOR ALPHA AND STAT3 SIGNALLING

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Thyroid hormones are essential for brain development, especially during the embryonic phase. However, the effects of thyroid hormone on the development of embryonic neural stem cells and the underlying mechanisms are poorly understood. Here, we found that insufficient of thyroid hormones during pregnancy inhibits telencephalon neurogenesis in mouse during embryonic phase, and destroys the learning and memory function. In our *in vitro* investigations, we treated embryonic day 13.5 mouse neural stem cells with physiological level of 3, 5, 3′-L-triiodothyronine. We found that 3, 5, 3′-L-triiodothyronine inhibits neural stem cell proliferation and maintenance, meanwhile promotes neuronal differentiation. In addition, thyroid hormone alpha was found expressing in embryonic neural stem cells both *in vivo* and *in vitro* and it is essential for the effects of 3, 5, 3′-L-triiodothyronine. Further studies found that 3, 5, 3′-L-triiodothyronine decreases tyrosine phosphorylation of STAT3 and subsequently inhibits STAT3-DNA binding activity. Over-expression of STAT3 can antagonize the effects of 3, 5, 3′-L-triiodothyronine. We also found the effects of thyroid hormone have no association with the ERK1/2 and Akt signallings, which are closely related to embryonic neural stem cell proliferation and differentiation. Taken together, these results suggest that thyroid hormone inhibits the proliferation and maintenance of embryonic neural stem cells and promotes neuronal dif-

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ferentiation through thyroid hormone receptor alpha and STAT3 signalling. These findings emphasize the importance of thyroid hormone functions during early brain development and suggest that embryonic neural stem cells are one of the major cellular targets of thyroid hormones.

Poster Board Number: 3446

INDUCTION EFFICIENCY OF SONIC HEDGEHOG ON DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS INTO NEURAL PRECURSOR CELLS WITH RNA EXPRESSION ANALYSIS

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Pluripotent embryonic stem cells (ESC) are capable to giving rise to derivatives of each of the three germ layer cells because of their ability to self-renew and differentiate into functional cells including neural cells. A major challenge at ESC researches is to develop novel processes for efficient *in vitro* cell type differentiation of ESC for analysis of gene function, and the therapeutic potential of using stem cells. Growth factors and cytokines promote *in vitro* differentiation and survival of ESC. If the *in vitro* differentiation processes of cells follows the development of neuroectodermal *in vivo*, we can expect to generate neural precursor cells can differentiate into both neural and glial cells. Sonic hedgehog (Shh) is a morphogenic protein that effects on ventral neuron induction and neural tube formation in early embryonic stages. We investigated enhancing neural differentiation of mouse ESC and inducing specific neural genes expression that control CNS development upon treatment with different concentrations of Shh. Moreover, for studying on differentiation efficiency of basic Fibroblastic Growth Factor (bFGF) we compared the effects of bFGF and Shh at the same time. Methods: Mouse ESC line was co-cultured on inactivated mouse embryonic fibroblasts and the resulting embryoid bodies were transplanted on culture plates that covered by poly- L- lysine. Shh was added to specific neural precursor cells by different concentrations, 300 and 500 nano molar (nM). Nestin (marker of neuroepithlial cells), Nkx2.2 (ventral neurons in spinal cord andV3 neurons), nurr1 (primary dopaminergic neurons), S100 (Astrocysts), Olig-2(oligodentrocytes and primary motor neurons), were selected to study the differentiation efficiency. Expression of these specific neural genes were assessed by RT-PCR and densitometry.In addition, the formation of dendrites and soma in neurons was assessed through immunocytochemistry assay on Microtubule Association Protein 2 (MAP2). Results: The expression of Nestin and Nkx2.2 showed Shh caused neural induction and ventral neurons in spinal cord differentiation. Nkx2.2 positive cells became more induction by 300 nm concentration of Shh. Moreover, bFGF 20 nM was added to the EBs culture and it showed synergic and special effects with Shh on neural induction and oligodendrocytes differentiation and the primary dopaminergic neuron formation that express nurr1 gene. Some of ES cells under treatment with Shh express Olig2 and were accordingly proved to be oligodendrocytes. However expression of s100 was not demonstrated and consequently no differentiation into astrocystes had happened. Immunocytochemistry assay of MAP-2 confirmed the formation dendrites and neurons. Conclusion: Our results suggest that Shh as a morphogenic protein induced *in vitro* neural gene expression and consequently it has positive effects on enhancing neural differentiation of ESC that could be employed in both neural development studies and may be future studied used for clinical trials.

Poster Board Number: 3448

CYSTEINE CATHEPSINS INDUCES THE DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS INTO NEURONS

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Embryonic stem (ES) cells are used as a model to understand the role of different molecules in embryonic development. Cystatin C (CysC), an endogenous inhibitor of cysteine cathepsins, was shown to induce the differentiation of mouse ES (mES) to neural stem cells. However, it is unclear whether CysC is acting as a cathepsin inhibitor or as a co-factor of bFGF. We investigated the possible role of cysteine proteases (CP) in neural differentiation by treating embryoid bodies (EB) from mES cells with: i) E64; an inhibitor of papain-like CPs and of calpains; ii) an inhibitor to cathepsin L; iii) an inhibitor to calpains; or iv) with cystatins, and assessed their ability to differentiate into neurons. After 24h treatment, E64 induced an increase in Pax6 expression in EBs. The inhibition of cysteine cathepsins, but not of calpains, led to a significant increase in the numbers of nestin-positive cells, three days later, indicating the induction of EB differentiation into neural progenitor cells (NPC). Glycosylated mouse CysC (mCysC), but not unglycosylated egg white cystatin (eCys), also increased the numbers of NPC. However, this effect was independent of CP inactivation. Neutralizing antibodies to bFGF blocked the induction of NPC only in mCysC-treated EBs, suggesting that bFGF is required for mCysC induced neural differentiation. Addition of bFGF increased the numbers of NPC in cultures derived from untreated EBs, or from eCys-treated EBs, but not from mCysC-treated EBs. Next, we followed cell differentiation for additional 24 days. In 14 days, we observed increased numbers of β-3-tubulin positive cells in populations derived from E64-treated EBs, showing greater percentage of immature neurons, a feature that persisted up to 24 days. At this stage, we verified the presence of functional neurons by performing electrophysiological records to detect Na+ channels. All cell populations displayed voltage-gated inward and outward currents when stimulated with depolarizing voltage steps. In cell populations originated from E64-treated EBs, we observed a significantly higher percentage of functional neurons (60%; n=10) as compared to untreated controls (20%; n=20). We propose that the inhibition of cysteine cathepsins induces the differentiation of mES cells into neurons, through a mechanism that is distinct from CysC-induced neuronal differentiation. This work was supported by: Edital CT-Biotecnologia/MCT/CNPq/MS/SCTIE/DECIT, CAPES, FAPERJ e INCT.

Poster Board Number: 3450

MESODERM AND ENDODERM DIFFERENTIATION DEFECTS OF PORCUPINE HOMOLOG MUTANT MOUSE EMBRYONIC STEM CELLS AND EMBRYOS

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In mammals, 19 Wnt ligands activate canonical and non-canonical pathways, which play important roles in development and disease. While research efforts have focused on the cells that receive Wnt signal, the Wnt secreting cells have not been extensively studied. Conserved from worms to human, the X-chromosomal gene porcupine homolog (Porcn) is a member of the membrane bound O-acyl transferase (MBOAT) superfamily.

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Biochemical evidence shows that Porcn is required for lipid modification of several, if not all Wnt ligands, which is essential for Wnt secretion or function. Embryonic lethality of several single Wnt mutants suggests a requirement for Porcn in mouse embryonic development. Using mouse embryonic stem (ES) cells carrying a genetrap null allele as well as a mouse line carrying a conditional allele, we present *in vivo* and *in vitro* evidence for the requirement of Porcn and Wnt signaling in early embryonic development and ES cell differentiation. Autocrine Tcf/Lef-Luciferase reporter assays show that Porcn null cells overexpressing Wnt3a exhibit defects in the secretion of functional Wnt3a ligand and absence of canonical Wnt signaling response. Further, paracrine reporter assays show that Porcn is exclusively required in the Wnt3a secreting cell, but not in Wnt signal receiving cells. *In vitro* differentiation of ES cells as embryoid bodies (EBs) in serum-free conditions demonstrates a requirement for Porcn in the generation of Flk1+ mesoderm and CXCR4+ endoderm after 4 days of differentiation. Quantitative real-time PCR analysis of EBs at 0, 2 and 4 days of differentiation suggests successful epiblast transition of Porcn null cells and confirms defects in the generation of mesodermal and endodermal tissues, based on the strongly reduced expression of Brachyury, Tbx6, Mixl, Sox17 and Cer1. Canonical Wnt signaling targets Axin2 and c-Myc were also significantly downregulated. Pluripotency and neural marker genes remain largely unchanged. Similar to the *in vitro* phenotype, aggregation of Porcn null ES cells with wildtype morula embryos as well as zygotic deletion of Porcn is lethal due to gastrulation defects *in vivo*. Using *in situ* hybridization at embryonic day 7.5 and 8.5, we detected prolonged expression of epiblast markers Oct4 and Otx2 throughout the embryo proper and a strong reduction or absence of anterior and posterior marker genes, as well as canonical Wnt signaling targets. These gene expression patterns as well as the embryonic morphology is reminiscent of the earliest single Wnt knock-out phenotype (Wnt3) and suggests a role for Porcn in the function of Wnt3, but does not exclude a role for other Wnts. Our data demonstrate a critical role for Porcn in canonical Wnt signaling, ES cell differentiation and mouse embryonic development.

Poster Board Number: 3452

SITE-SPECIFIC DIFFERENTIATION OF MURINE EMBRYONIC STEM CELL-DERIVED VASCULAR PROGENITORS BY IMMOBILIZED GROWTH FACTORS FOR VASCULAR TISSUE ENGINEERING

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Engineered tissue patches have the potential to repair and replace damaged and diseased tissues. However, mass transfer limitations require embedding engineered blood vessels in the tissue to achieve physiologically-relevant tissue thickness. Vascular progenitor cells can be differentiated into vascular endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) in a site-specific manner by manipulating adhesion substrates and growth factor concentrations on a surface. We showed previously that vascular endothelial growth factor (VEGF), when covalently immobilized onto CollagenIV (ColIV) using EDC chemistry, was capable of differentiating murine embryonic stem cell (mESC) derived Flk1+ vascular progenitors toward a CD31+ EC phenotype, on both patterned and uniform surfaces. However, we have not elucidated the effects of PDGF, and whether having the two growth factors on the same surface would elicit differential responses in differentiation. In addition, the immobilization techniques used could potentially release growth factors in soluble form due to substrate degradation. Growth factors immobilized on a non-degradable surface would be necessary to ascertain that the differentiation was due to the immobilized growth factor and not due to degradative release. Here, we demonstrated that not only did these Flk1+ vascular progenitors yield primarily CD31+ ECs when cultured on surfaces with immobilized or soluble VEGF, they yielded mostly SMA+ VSMCs when cultured on surfaces with PDGF (immobilized or soluble) or ColIV alone. The immobilized growth factors had a dose-responsive effect on differentiation. Decreases in VEGF concentration resulted in corresponding decreases in EC differentiation, whereas decreases in PDGF concentration resulted in increases in EC differentiation. Differential responses could be observed when both growth factors were immobilized onto the same surface. ECs were observed on the VEGF-immobilized area, whereas VSMCs were found on the PDGF-immobilized area. In addition, Flk1+ progenitors seeded onto non-degradable surfaces with GMBS-immobilized VEGF and PDGF showed similar responses to soluble controls and EDC-immobilized conditions. Quantitative PCR analysis showed significant down-regulation of Oct4 and up-regulation of Flk1. EC and VSMC markers were differentially expressed in VEGF and PDGF conditions. CD31 expression was higher in VEGF conditions while SMA and Calponin expression were higher in PDGF conditions. Together, these results demonstrated that differentiation is attributed largely to immobilized growth factors, not the degradative release of growth factors into media. ECs and VSMCs can be reproducibly generated from a common progenitor by immobilizing and patterning growth factors to direct differentiation in a site-specific manner. Ongoing and future work will focus on human ESC-derived cardiovascular progenitors.

Poster Board Number: 3454

NOTCH SIGNALING DEFINES TWO DISTINCT HEMATOPOIETIC PROGRAMS IN MOUSE EMBRYONIC STEM CELL DIFFERENTIATION CULTURES

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The requirement for Notch signaling is one characteristic that distinguishes yolk sac from para-arotic splanchnopleura (P-Sp)/aorta-gonad-mesonephros (AGM) hematopoiesis in the mouse embryo. Whereas this signaling pathway is dispensable for generation of the yolk sac blood cell lineages, it is essential for establishment of the intraembryonic P-Sp/AGM hematopoietic program and the formation of the first hematopoietic stem cells (HSCs). We have recently identified two temporally distinct Flk1+ hematopoietic populations in mouse embryonic stem cell (ESC) differentiation cultures that display characteristics of yolk sac and P-Sp/AGM hematopoiesis. When induced with the combination of activin A, BMP-4 and VEGF in an embryoid body (EB) format, mESCs generate a Flk-1+ population within 72 hours of differentiation that displays the capacity to give rise to primitive erythrocytes. This early Flk-1 population has little T-cell potential. When cultured for an additional 48 hours, the day 3.0 EB cells give rise a second Flk-1+ population that shows diminished primitive erythroid potential and enhanced capacity to generate T-lymphocytes compared to the early population. To determine if Notch signaling distinguishes these two waves of hematopoiesis in the mESC cultures, we analyzed the hematopoietic potential of the two Flk-1 populations generated from RBP-Jk null cells that are unable to signal through this pathway. As expected from previous studies, the hematopoietic potential of the early arising Flk-1+ population is enhanced showing an increase in the frequency of primitive erythroid progenitors compared to controls. In contrast, the second Flk-1+ population derived from the RBP-Jk null cells showed dramatically reduced hematopoietic potential. Taken together, these findings demonstrate that the two ESC-derived hematopoietic populations differ in their requirement for Notch signaling and as such, provide strong support for the hypothesis that they represent the *in vitro* equivalent of the yolk sac and P-Sp/AGM-derived hematopoietic programs.

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THE NOTCH SIGNALING PATHWAY, AN IMPORTANT REGULATOR IN MOUSE EMBRYONIC STEM CELLS DERIVED HAEMATOPOIESIS *IN VITRO*

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Mouse Embryonic Stem Cells (mESCs) are able to differentiate into many cell lineages *in vitro*, including all haematopoietic cell types. The aorta-gonad- mesonephros (AGM) region is the first site in the embryo where haematopoietic stem cells (HSCs) are generated and is likely to be rich of haematopoietic inductive signals. We have demonstrated previously that the production of haematopoietic progenitors (HPCs) from both mouse and human ES cells is significantly enhanced when co-cultured with primary AGM and AGM-derived stromal cell lines (AM), which supplied a powerful platform for investigation the molecular mechanisms of haematopoiesis *in vitro*. We demonstrated that the haematopoietic enhancing effects of the ES/AGM-derived stromal cell co-culture system was significantly reduced when inhibition of the Notch signaling pathway was applied with γ-Secretase Inhibitor(GSI) after the formation of mesoderm. To further clarify the role of the Notch signaling pathway during haematopoietic differentiation of mESCs, we have assessed the activity of the Notch pathway throughout the AGM stromal/ES cell co-culture period and have established both gain- and loss-of-function tools. We have shown that the Notch pathway is active in ES cells when they are co-cultured with AGM-derived stromal cell lines but that the level of Notch activity did not correlate with the level of haematopoietic induction displayed by the different stromal cell lines. In a gain of function strategy we have used a ES cell line in which Notch Intracellular Domain (Notch-IC) expressing cells can be selected by flow cytometry: tamoxifen-induced CRE-mediated excision results in expression of both Notch-IC and the cell surface marker CD2. We have also generated a novel loss-of-function strategy to down-regulate Notch activity in mESCs by inserting a dominant-negative MAML (DNMAL)-EGFP fusion protein into the doxycycline-inducible A2loxCre system. The DNMAL protein inhibits Notch activity by inactivation of the RBP-Jκ complex. This inducible system will allow us to elucidate role of Notch in the mESCs at different time points during the co-culture period. To date, the expression of the DNMAL-EGFP fusion protein has been validated by Western blotting and flow cytomtery and we have used the 12xRBP-Jk-luciferase reporter (12xRBP-Jκ-Luc) to confirm that the DNMAL-GFP fusion protein was able to inhibit Notch activity upon addition of doxycycline. We are currently assessing the effects of NOTCH-IC and DNMAL expression on the production of haematopoietic progenitors from FLK1+ cells using methylcelluose assays and surface marker expression (CD41, c-kit and CD45).

Poster Board Number: 3458

HOXB4 CAN ENHANCE THE DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS BY MODULATING THE HAEMATOPOIETIC NICHE

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Haematopoietic differentiation of mouse embryonic stem (ES) cells *in vitro* is a powerful tool to model early blood cell development and to generate cells for regenerative medicine. A number of strategies have been used to increase the production of haematopoietic cells from ES cells and to enhance the functional properties of the haematopoietic progenitors that are generated. These include co-culture on a variety of stromal cell lines and over-expression of homeobox transcription factors such as HOXB4. To more fully understand the mechanism of action of HOXB4 we established ES cell lines carrying a HOXB4-ERT2 fusion protein that allows the activation of HOXB4 at defined time points during the differentiation process. We demonstrate that the activation of HOXB4 prior to the emergence of haematopoietic progenitors results in an increase in haematopoietic differentiation suggesting that HOXB4 acts on the pre-haematopoietic mesoderm. An increase in haematopoietic differentiation was observed when GFP+ ES cells were cultured in the presence of HOXB4-ERT2-expressing ES cells indicating that HOXB4 activation has a paracrine, as well as a cell autonomous, effect on haematopoietic differentiation. This hypothesis was supported by expression profiling of HOXB4 target genes at early stage of ES cell differentiation that identified a number of genes associated with paraxial mesoderm that gives rise to the haematopoietic niche. We provide evidence that this paracrine effect may, in part, be mediated by modulation of the Wnt pathway.

Poster Board Number: 3460

THE ROLE OF SOX17 IN THE SPECIFICATION OF EARLY HEMATOPOIETIC PROGENITORS FROM MOUSE EMBRYONIC STEM CELLS

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The capacity of embryonic stem cells (ESCs) to generate differentiated progeny provides a model system for studying mammalian development as well as a source of cells for pharmaceutical and therapeutic applications. Within this context, the generation of hematopoietic stem cells (HSCs) is of particular interest, as the derivation of these cells would provide a novel source of stem cells for transplantation and for modeling hematological diseases *in vitro*. To achieve this goal, it is necessary to recapitulate embryonic hematopoiesis in stem cell cultures. Embryonic hematopoiesis is temporally and spatially regulated; the first wave of primitive hematopoiesis occurs in the yolk sac (YS) followed by definitive hematopoiesis and the emergence of the HSC in the para-aortic splanchnopleura (P-Sp). Lineage tracing studies have shown that both hematopoietic programs develop from Flk-1+ progenitors cells. To recapitulate YS and P-Sp hematopoiesis in ES cell cultures we identified two temporally distinct Flk-1+ hematopoietic progenitor populations that showed different developmental potential. The earliest Flk-1+ population contained large numbers of primitive erythroid precursors and few lymphoid precursors whereas the later arising Flk-1+ population displayed lymphoid potential and diminished primitive erythroid potential. We hypothesized that these Flk-1+ progenitors represent the equivalent of

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the yolk sac and P-Sp progenitors respectively. Further analysis of the Flk-1+ P-Sp population revealed the expression of Sox17 enriches for lymphoid potential and that VEGF and TGFβ signaling are required for Sox17 expression. Additionally, analysis of E9.0 mouse embyros confirmed that Sox17 expression is enriched in Flk-1+ cells. Enforced expression of Sox17 in Flk-1+ cells induced an endothelial cell type that maintains hematopoietic potential suggesting that Sox17 may play a role in the generation of hemogenic endothelium. Taken together these findings suggest that Sox17 plays a role in the generation of definitive hematopoietic progenitors in ES cell cultures.

Poster Board Number: 3462

POLYCOMB GROUP PROTEIN BMI1 PROMOTES HEMATOPOIETIC CELL DEVELOPMENT FROM MOUSE ES CELLS

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Bmi1 is a component of the Polycomb-repressive complexes (PRC) and essential for maintaining the pool of adult stem cells. PRC are key regulators for embryonic development by modifying chromatin architecture and maintaining gene repression. To assess the role of Bmi1 in pluripotent stem cells and upon exit from pluripotency during differentiation, we studied forced Bmi1 expression in mouse embryonic stem cells (ESC). We found that ESC do not express detectable levels of Bmi1 RNA and protein and that forced Bmi1 expression had no obvious influence on ESC self-renewal. However, upon ESC differentiation Bmi1 effectively enhanced development of hematopoietic cells. Global transcriptional profiling identified a large array of genes that were differentially regulated during ESC differentiation by Bmi1. Importantly, we found that Bmi1 induced a prominent up-regulation of Gata2, a zinc finger transcription factor, which is essential for primitive hematopoietic cell generation from mesoderm. In addition, Bmi1 caused sustained growth and a more than 100-fold expansion of ESC-derived hematopoietic stem/progenitor cells within 2-3 weeks of culture. The enhanced proliferative capacity was associated with reduced Ink4a/Arf expression in Bmi1-transduced cells. Taken together, our experiments demonstrate distinct activities of Bmi1 in ESC and ESC-derived hematopoietic progenitor cells. In addition, Bmi1 enhances the propensity of ESC in differentiating towards the hematopoietic lineage. Thus, Bmi1 could be a candidate gene for engineered adult stem cell derivation from ESC.

Poster Board Number: 3464

A COLLAGEN-BASED MATRIX THAT ENHANCES CARDIAC AND SKELETAL MYOGENESIS IN MOUSE EMBRYONIC STEM CELLS

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Statement of Purpose: It has been shown previously that a collagen matrix containing sialyl Lewis X (sLex), an L-selectin ligand found on CPCs, can enhance the regeneration of ischemic muscle1, and accelerate the differentiation of C2C12 myoblasts. In this study, we tested whether or not these matrices would also support the formation of a myogenic progenitor population from mouse embryonic stem cells (ES). This population should replenish the satellite cell niche when engrafted into muscle. Considering the difficulty of grafting stem cells *in vivo*, a matrix that can support the formation of myogenic progenitor cells would be valuable as a potential method of delivery for future cell therapy approaches. The effects of a collagen matrix, with and without sLex, were evaluated during skeletal and cardiac myogenesis in mouse ES cells. Results: Skeletal muscle: Immunofluorescent staining identified skeletal myocytes on the collagen surface with a higher number of nuclei compared to the control surface, indicating enhanced cell fusion. This was confirmed by Q-PCR analysis of a skeletal muscle-specific fusion marker, nfatc3. Q-PCR analysis of skeletal muscle precursor markers showed a significantly higher expression of Pax3 and Pax7 in the cells differentiating on the collagen and sLex compared to control tissue culture plates, indicating enhanced myogenic progenitor formation. Furthermore expression of myogenic regulatory factors, Myf5 and Myogenin was significantly higher on both surfaces, indicating enhanced myoblast/myotube formation. Expression of the final differentiation marker MHC3 was also significantly higher for the matrix-cultured compared to the control. Cardiac muscle: It was observed that a greater number of cells differentiated into cardiac muscle on the sLex surface, as judged by the number of beating foci. When compared to the control surface, the beating of the cells on the sLex surface was more synchronized. Expression of the cardiomyogenic genes GATA4 and Nkx2.5 and the cardiac muscle-specific peptide, ANF, was significantly higher on the sLex surface, indicating enhanced cardiomyogenesis. Conclusions: These results demonstrate that the collagen matrix enhanced skeletal myogenesis, indicated by increased numbers of multinucleated myotubes and upregulation of skeletal muscle markers. The sLex -modification of the collagen matrix enhanced cardiomyogenesis in mouse ES cells, shown by increased beating and upregulation of cardiogenic genes. Thus we have identified biomaterials that could serve a two-fold purpose of providing an injectable substrate for delivery of stem cells and enhancing the progenitor population of both cardiac and skeletal muscles.

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CELL-SECRETED SOLUBLE FACTORS INDUCE ENHANCED DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS TOWARDS MESODERM AND ENDODERM IN A MEMBRANE-BASED MICRO-BIOREACTOR CULTURE

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Mimicking *in vivo* situation *in vitro* enables better understanding and control on embryonic stem cell fate choice. At the initial stage of development, pluripotent stem cells in the embryo change their fate under the influence of autocrine/paracrine signaling in a diffusion dominant microenvironment. However, in the static open macro-scale culture (for example, Petri dish) systems, cell secreted soluble factors swept away from cell neighborhood through spontaneous convection as well as become diluted in large volumes as compared to the enclosed micro-scale culture systems. Therefore, we utilized a membrane-based two-chambered micro-bioreactor (MB) to differentiate mouse embryonic stem cells (mESCs) in a diffusion dominant microenvironment. Common techniques of micro-fabrication were used to fabricate the MB. Both chambers heights and volumes were 500 μm and 114 μL, respectively. Chambers were kept separated by the semipermeable membrane. mESCs were differentiated for 8 days on the membrane side facing upper chamber of the MB in static condition. Culture medium containing Knockout Serum (KSR, Gibco),Knock-out DMEM (Gibco), and other basic constituents was used for the differentiation. Two operation conditions were used to differentiate mESCs in the MB: culture medium from 1) both chambers (MB1), and 2) only the lower chamber (MB2) was exchanged after every 2 days. In the MB1, differentiated cells at day 8 formed an epithelial sheet-like morphology. Differentiated cells in the MB2 also formed epithelial sheet-like morphology; however, it was not extensive as MB1. In contrast to the MBs, differentiated cells in the 6-Well plate (6-WP) formed

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spherical colonies. Some of these colonies had neural extension spreading out from them. Genes related to pluripotency and different germ layers also showed different expression level in the culture systems. Expression level of pluripotency markers showed that, loss of pluripotency in the MB1 was higher whereas it was lower in the MB2 as compared to the 6-WP. Expression level of ectoderm related genes showed no difference among culture systems. However, expression levels of mesoderm and endoderm related genes were higher in the MB1 and MB2 than the 6-WP. Notably, mesoderm related gene expression was highest in the MB1 than MB2 though endoderm related genes showed no significant difference between the two. Between the soluble factors Fgf4 and Bmp4, Fgf4 expression was lower in the MB1 than both 6-WP and MB2. However, Bmp4 expression was higher both in the MB1 and MB2 than the 6-WP. Presumably, differential effects of Fgf4 and Bmp4 in the MB1 and MB2 might have influenced the differentiation behavior of mESCs. Currently, we are investigating the effects of these soluble factors through their inhibition experiments in the MB1 and MB2. Our study demonstrated that, mESCs differentiated more extensively in the MB1 than conventional macro-scale culture. In addition, medium exchange from the chambers (influencing the accumulation of the soluble factors) had also affected the mESC differentiation profile. The study also showed that, in the absence of any exogenous soluble factors, mESCs can differentiate not only towards ectoderm but also towards mesoderm and endoderm through the autocrine effects of soluble factors in an environment provided by the micro-bioreactor. Therefore, the microbioreactor can be utilized to derive mesodermal or endodermal cells which derivations from mESCs are not straight forward as neural cells.

Poster Board Number: 3468

IDENTIFICATION OF MOUSE EMBRYONIC STEM CELL FATE DETERMINANTS

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We have developed a viral based Cre/LoxP strategy that efficiently creates chromosomal deletions in mouse embryonic stem (ES) cells. Using this method, we generated a collection (named DelES) of more than 1000 clones carrying a chromosomal deletion. An *in vitro* embryoid body (EB) formation screen was conducted with DelES clones to assess differentiation problems throughout our collection. Our aim is to complement these phenotypes by reinserting DNA fragments into the deleted clones, thus potentially identifying new determinants of ES cell fate. Complementation studies of DelES family 9 showed that only one BAC was able to rescue the *in vitro* EB formation phenotype caused by a 4.3Mb deletion. Additional experiments identified the ribosomal protein S14 (Rps14) coding gene as being haploinsufficient for EB formation. Altered ribosomal proteins expression has been shown to induce a "nucleolar stress" which results in increased levels of the p53 protein. Western blot analyses revealed that p53 was more abundant in family 9 clones. Interestingly, an shRNA against p53 was able to rescue the differentiation defect of clone 9-37. This result suggests that Rps14 is essential for EB formation and that its deletion leads to a p53-related block of differentiation. Our latest studies on ribosomal proteins confirmed that a p53 block of differentiation was common in at least one other family of clones. Future studies will focus on the molecular events that are responsible for the differentiation phenotype seen upon ribosomal protein defects. Moreover, about 30 ribosomal protein genes are deleted in different DelES families. It will therefore be interesting to compare the role of different ribosomal proteins in ES cells differentiation.

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"RESIDUAL POTENTIAL", THE NEW CHARACTERISTIC OF MOUSE EMBRYONIC STEM CELLS

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Embryonic stem (ES) cells are a potential cell source for regenerative medicine. However, the residual undifferentiated embryonic stem (rES) cells after *in vitro* differentiation are tumorigenic during transplantation. These cells are thought to be genetically mutated cells due to long-term culture. Here, we proposed that generating rES cells during differentiation might be a unique characteristic of ES cells which could protect themselves. To prove this hypothesis, an *in vitro* and an *in vivo* ES cell differentiation system were utilized to characterize the residual cells during differentiation. We demonstrated that rES cells existed in both embryoid bodies (EBs) and teratomas and were indistinguishable from their parental cells. The rES cells could be re-differentiated to form EBs *in vitro* and form teratomas *in vivo*, and importantly, could participate in normal mouse development following blastocyst injection, indicating that they are normal pluripotent cells rather than mutant cells. Further more, we proved that the primary cultured ES cells were heterogeneous, with few cells having the "residual potential" that could generate rES cells during differentiation, and the rES cells were enriched in the PECAM-1 and Oct-4 double positive population in long-term EBs and teratomas. This is the first study to demonstrate that a small number of normal mouse ES cells could be maintained in a pluripotent state during differentiation. This new characteristic of mouse ES cells prompts us to reconsider the strategies for solving the tumorigenic problem of stem cells during cell therapies.

Poster Board Number: 3472

EXPRESSION OF HAEMATOPOIETIC STEM CELL MARKERS C-KIT AND SCA-1 RELATES TO CELL CYCLE STATUS

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INTRODUCTION: Previous studies have investigated whether levels of expression of the haematopoietic stem cell (HSC) marker C-kit relates to cell cycle status in sorted populations enriched for HSCs. These studies have found no link although C-kit deletion mutant mice do show significantly reduced HSC numbers. Sca-1 deficient mice have a reduction in immature haematopoietic progenitors and HSCs show impaired repopulating ability. METHOD: We investigated haematopoietic stem cell marker expression in unsorted cell populations at different developmental stages including differentiating ES cells, foetal liver cells and adult bone marrow cells. Flow cytometry studies were performed to determine expression of C-kit, Sca-1 and other HSC markers in these different populations. Bone marrow populations were then sorted based on expression of C-kit or Sca-1 and analysed for cell cycle status. RESULTS: We find higher levels of C-kit expressing cells in day 6 ES cells and foetal liver cells than in bone marrow cells ($P<0.05$). However there are higher levels of Sca-1 expressing cells in bone marrow cells compared with foetal liver cells ($P<0.05$). In development many more cells are dividing and we hypothesised that expression of C-kit is related to cell division. Given higher levels of Sca1 expression in the adult we hypothesised that Sca-1 expression is related to quiescence. Further experiments reveal that in the bone marrow 83% of Sca-1 expressing cells are in G1/0 resting phase of the cell cycle ($P<0.05$). However in the bone marrow derived C-kit population 60% of cells were in G2/M or S phase ($P<0.05$). CONCLUSION:

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This initial data indicates that C-kit is expressed on dividing cells and Sca-1 is expressed on cells that are quiescent. We propose that previous experiments have not demonstrated this as they have focused on levels of marker expression and cell cycle status in specific HSC populations that may select out cells in a particular stage of the cell cycle.

Poster Board Number: 3474

NEW PHYSIOLOGICAL DIFFERENTIATION SYSTEM TO DERIVE HIGH-PURITY DEFINITIVE ENDODERM LINEAGES UNCONTAMINATED BY UNDIFFERENTIATED CELLS

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Derivation of differentiated cells uncontaminated by undifferentiated cells that may evolve into tumors is one of a major objective of stem cell research. We report here a novel physiological approach to derive from various human pluripotent stem cell types, a high-purity definitive endoderm (DE) and DE lineages uncontaminated by undifferentiated cells. Our new differentiation culture system reproduces *in vitro* the conditions encountered by cells during gastrulation and differentiation into functional cell types *in vivo*. Detailed description of the new culture system will be presented. Our observations indicate that the cell type derived in new differentiation system by the end of DE-stimulation step of the differentiation protocol is an authentic DE. Marker analysis at the protein and RNA levels was consistent with the formation of DE. Flow cytometry data analysis indicated the extremely high purity of these cultures and no undifferentiated cells were detected in the created DE. Reverse transcriptase (RT) real-time quantitative PCR revealed a lack of distinct expression of mesoderm, ectoderm, trofectoderm as well as extraembryonic endoderm markers. Further direct differentiation of cells toward hepatocytes within our differentiation system produced cells manifesting the same cubical shape as mature hepatocytes, and expressing a variety of genes and proteins, typical for functional mature hepatocytes. Comparative analysis of hepatocyte-like cells derived in our differentiation system from human pluripotent stem cells, and mature human hepatocytes was based on RT real-time quantitative PCR gene detection. To determine whether the test cells express metabolic functions characteristic of normal human hepatocytes, we examined the activities of several phase I (CYPP450) and phase II drug-metabolizing enzymes by using reversed-phase HPLC; expression of the appropriate drug-methabolizing enzymes was additionally confirmed by RT real-time quantitative PCR. The purity of derived hepatocytes was determined by FACS analysis. Obtained data will be presented and discussed. The efficacy of this method was demonstrated for both human embryonic and human parthenogenetic stem cells. The latter cell type holds a great promise as a source of pluripotent stem cells for cell-based transplantation therapy due to the ethical method of derivation, as well as the enhanced capacity for immune-matching with significant segments of human population. Thus, we postulate that, if culture of pluripotent stem cells can respond to direct differentiation signaling and can produce DE lineages, our system will be an effective new approach to isolate a high-purity population of DE lineages. The ability to work with a wide range of cell lines may also facilitate and promote cell transplantation based on stem cells derived from the patient or histocompatible donors. The method described here represents a significant step toward the efficient generation of high purity hepatocytes and pancreatic endocrine cells for use in regenerative medicine and drug discovery.

Poster Board Number: 3476

SKELETAL MYOSIN LIGHT CHAIN KINASE REGULATES SKELETAL MYOGENESIS BY PHOSPHORYLATION OF MEF2C

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The MEF2 transcription factors play an important regulatory role in several lineages. In skeletal myogenesis, MEF2 factors amplify and synergize with MyoD, establishing myoblast formation. While phosphorylation is known to regulate MEF2 function, lineage-specific regulation is unknown. Here, we show that a novel phosphorylation of MEF2C on T80 by skeletal myosin light chain kinase (skMLCK) enhances skeletal and not cardiac myogenesis. A phosphorylation-deficient MEF2C mutant (MEFT80A) was able to enhance cardiac, but not skeletal myogenesis in P19 stem cells. Further, MEFT80A was deficient in recruitment of p300 to skeletal but not cardiac muscle promoters. In gain-of-function studies, skMLCK enhanced skeletal myogenesis in P19 cells and myogenic conversion assays. In loss-of-function studies, MLCK was essential for efficient myogenesis in P19, mouse embryonic stem (ES) and mouse progenitors cells. Our findings define a novel function for skMLCK in regulating myoblast formation, by controlling the MEF2C-dependent recruitment of histone acetyltransferases (HATs) to skeletal muscle promoters, going beyond the known role of skMLCK in muscle contraction. Finally, we identify the first kinase that regulates skeletal myogenesis in ES cells.

Poster Board Number: 3478

STUDIES OF MESP1 IN EARLY DEVELOPMENT OF MESODERMAL LINEAGES

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Recently we and others identified the gene mesoderm posterior 1 (Mesp1) as an important regulator of early cardiovascular fate specification during differentiation of murine embryonic stem cells (ESCs). We observed that induction of Mesp1 during early ESC differentiation could drive a program of epithelial-mesenchymal transition (EMT) that was independent of Wnt signaling, in contrast to spontaneous EMT which required Wnt signaling. To identify how Mesp1 directs EMT, we sought to identify its direct targets during ESC differentiation. Microarray analysis identified PDGFRα, being increased 14-fold within 6 hours of Mesp1 induction in differentiating ESCs. PDGFRα induction by Mesp1 may be important, since PDGFR signaling plays a role in normal gastrulation, the survival of mesodermal cells and the induction of mesoderm during embryogenesis. We tested whether PDGFRα is a direct target of Mesp1, and the possibility that it mediates Mesp1's functions during ESC differentiation. Here we report that Mesp1 directly binds to the PDGFRα promoter in differentiating ESCs by EMSA. In addition, by inducing Mesp1, but blocking PDGFRα signaling through use of specific PDGFR inhibitors, we show that PDGFRα signaling is also necessary for Mesp1's effects in cell survival, EMT and mesodermal cell fate specification. Another unanswered question is how Mesp1 functions in the specification of cardiovascular lineages. In examining ESC differentiation, we found that lineage segregation between endothelial and mesenchymal progenitors, which generate smooth muscle cells (SMC) and cardiomyocytes, occurred as early as 2 days after Mesp1 induction. Factors that are differentially expressed between these endothelial and mesenchymal progenitors at this early stage may interact with Mesp1 to specify individual cardiovascular cell lineage. To identify such potential factors, we isolated purified populations of differen-

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tiating ESCs that that appear to be endothelial progenitors (Flk1hiTie2+) or mesenchymal progenitors (Flk1medPDGFRα+Tie2-) from Mesp1-induced ESCs and compared their gene expression by global microarray analysis. The transcription factors Erg and Fli-1 were enriched in endothelial progenitors. We show that these factors promote endothelial differentiation in differentiating ESCs. Fli-1 also partially inhibited smooth muscle differentiation. In contrast, Baf60c, a subunit of the Swi/Snf-like BRG1-associated factors (BAF) complex, was enriched in mesenchymal progenitors. Knockdown of Baf60c in differentiating ESCs reduced induction of cardiac troponin T (cTnT) by Mesp1, suggesting that Mesp1 requires Baf60c to induce cardiac lineages. Our studies further elucidate the mechanisms by which Mesp1 regulates EMT and early cardiovascular specification, which is important for understanding cardiac development and eventual applications to regenerative therapy.

Poster Board Number: 3480

SCREENING SMALL MOLECULES FOR INDUCERS OF CARDIOMYOCYTE CELL LINEAGE FATE

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Chemical genetics has become an increasingly robust and general paradigm for addressing difficult biological questions. This approach has been applied in the field of regenerative medicine leading to the identification of a number of small molecules used to control stem cell fate. Our current focus is on identifying and characterizing small molecules that can induce human pluripotent cells to differentiate into cardiomyocytes. Because kinases are likely to play an important role in cell signaling processes that regulate stem cell proliferation and differentiation, a combinatorial library designed around kinase-directed molecular scaffolds was screened to identify members that differentiate human embryonic stem cells (hESCs) into cardiomyoctes. We are currently characterizing the mechanism of the identified compounds by determining the target(s) using affinity based approaches and investigating the signaling pathways using genetic and biological methods.

Poster Board Number: 3482

FBXO25 ACTS AS AN E3 LIGASE TO TARGET TBX5 AND HAS A PROFOUND EFFECT ON CARDIOMYOCYTE DIFFERENTIATION IN EMBRYONIC STEM CELLS

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Fbxo25 is a subunit of functional ubiquitin ligase complex containing Skp1, Cul1, and Rbx1. Tbx5 is one of transcription factors which regulate formation of embryonic heart. Mutations in this gene have been known to associate with Holt-Oram syndrome, a developmental disorder affecting the heart and upper limbs. Here we show that Fbxo25 bound to Tbx5 and promoted Tbx5 degradation. Tbx5 associated with N-terminal domain in Fbxo25. Fbxo25 bound to T-box domain of Tbx5. Tbx5 degradation was triggered by ubiquitination through SCF complex with Fbxo25. In addition, Tbx5 increased the activities of atrial natriuretic peptide (ANP)-luciferase reporter, but Fbxo25 decreased the effects of Tbx5 on ANP-luciferase in parallel with degradation of Tbx5 proteins. We performed cardiomyocyte differentiation assays using embryonic stem cells (ESCs) expressing wild type or dominant negative Fbxo25 mutants. As a result, the ratios of cardiomyocyte differentiation were dramatically compromised compared to control ESCs. The expression of Fbxo25 using Fbxo25 lenti-viruses decreased Tbx5 expression in the regions of myocardiac infarction. These results indicated that Fbxo25 has an important role in cardiac development and differentiation through Tbx5 degradation.

Poster Board Number: 3484

EMBRYONIC STEM CELL-DERIVED RENEWABLE AND FUNCTIONAL MIDBRAIN DOPAMINERGIC PROGENITORS

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During early development, midbrain dopaminergic (mDA) neuronal precursors (NPs) arise from the ventral mesencephalic area by the combined actions of secreted factors (e.g., Shh and Wnt 1) and their downstream key transcription factors. These mDA NPs proliferate, migrate to their final destinations, and develop into mature mDA neurons in the substantia nigra and in the ventral tegmental area. Here, we show that such authentic mDA NPs can be efficiently isolated from *in vitro* differentiated mouse embryonic stem cells (ESCs) using a fluorescent activated cell sorting (FACS) method combining two-markers, Otx2 and Corin. Purified Otx2+ Corin+ cells coexpressed other mDA NP markers such as FoxA2, Lmx1b and Glast. Using our optimized *in vitro* culture conditions these mDA NPs continuously proliferated for at least up to 4 weeks with almost 1,000-fold expansion without significant changes in their phenotype and developmental potential. Furthermore, upon neuronal differentiation, Otx2+ Corin+ cells efficiently generated mDA neurons, as evidenced by coexpression of mDA neuronal markers (e.g., TH, Pitx3, Nurr1 and Lmx1b) and physiological functions (e.g., efficient DA secretion and uptake). Notably, these mDA NPs differentiated into a relatively homogenous population containing high DA neurons but very few serotonergic neurons. When transplanted into a mouse model of PD, aphakia mice, they differentiated into mDA neurons *in vivo* and generated well-integrated grafts with enriched DA neurons, resulting in significant improvement of motor dysfunctions without tumor formation. Furthermore, grafted Otx2+ Corin+ cells exhibited significant migratory function in the host striatum, reaching up to >3.3mm length in the entire striatum. We propose that functional and expandable mDA NPs can be efficiently isolated by this novel double sorting strategy and will serve as useful platforms in regenerative medicine, bioassay, and drug screening for DA neuron-related biology and diseases.

Poster Board Number: 3486

SURFACEOME PROFILING REVEALS NOVEL REGULATORS OF NEURAL STEM CELL FUNCTION

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The composition of cell-surface proteins changes during lineage specification, altering cellular responses to their milieu. Cell-autonomous properties arise including distinct adhesive profiles that facilitate morphogenetic movements, boundary formation towards organization of tissues as well as cell-intrinsic lineage programs which restrict the range and output of morphogen responsiveness. The prevailing model of neural induction posits that epidermis is induced from ectoderm by BMP-induced signaling, while

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neural tissue is segregated from ectoderm by release of BMP antagonists such as Noggin and Chordin from anterior visceral endoderm at E7.5 of murine development; BMP antagonism effectively relieves repression of neural fate, as factors downstream of BMPs including SMADs and IDs inhibit the neural program. These events in development can be modeled using pluripotent Embryonic Stem Cells that self-renew when cultured in serum-free media supplemented with BMP4 and Lif, but undergo neural induction to form Primitive Neural Stem Cells (pNSCs) when placed at low density in serum free media with Lif but without exogenous BMPs, and Definitive (dNSCs) when pNSCs are passaged into FGF-containing media without Lif. The changes that characterize maturation of early NSCs remain poorly understood, and improved characterization would help elucidate the regulators of critical NSC properties. We reasoned that an unbiased approach to examining the cell-surface constituents of each cell in the lineage of early NSCs would provide direct insight into molecules that functionally dictate the unique identity of each cell type. Here we use Mass-Spectrometry based Cell Surface Capture technology to profile the cell surface of early NSCs and find many molecules enriched on the cell surface of one of ES cells, pNSCs or dNSCs, and not on the other two cell types. Moreover, we demonstrate functional requirements for several of the enriched molecules selectively on each of the two NSC types. Specifically, we demonstrate that Neural-cadherin is the predominant determinant of dNSC compartmentalization, c-kit signaling promotes pNSC quiescence, signaling through EphA4 is required for viability of both NSC populations and that Erbb2 signaling is required for the proliferation of pNSCs. This work elucidates several key mediators of NSC function whose relevance then is confirmed on forebrain-derived populations and identifies a host of other candidates that may regulate NSCs.

Poster Board Number: 3488

OLIGODENDROCYTES AND MOTONEURON PROGENITORS DERIVED FROM HESC RESCUE LOCOMOTOR FUNCTION IN RATS WITH COMPLETELY TRANSECTED SPINAL CORD

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Human embryonic stem cells (hESC) hold great promise in the treatment of many neurodegenerative human diseases particularly those arising from cell loss or neural dysfunction what is the case of spinal cord injury (SCI). This study is designed to evaluate the therapeutic effects on axonal remyelination and functional recovery of complete transacted spinal cord of transplanted hESC-derived oligodendrocytes (OPC) and/or motoneuron progenitors (MP). Adult female rats were subjected to the model of complete transection SCI and hESC- derived OPC and/or MP were grafted into the site of injury in the acute phase. Immunohistochemical data provide evidence differentiation of hESC derived OPCs and MPs into several neural phenotypes including neurons and oligodendrocytes. Recovery of hind limb locomotor function was also significantly enhanced in the rats treated with a single cell type (OPC or MP) or combination of these two cell types compared with the control lesioned rat treated with vehicle only, based on Basso-Beattie-Bresnahan (BBB) scores assessed during the 4 months after transplantation. We demonstrate that OPC and MP, when transplanted into the spinal cord immediately after complete transection survive and differentiate into mature oligodendrocytes and neurons and improve locomotor function. These results show that OPC and MP derived from hESC could be a useful therapeutic strategy to repair the injured spinal cord.

Poster Board Number: 3490

EXPRESSION AND FUNCTION OF A TYPE II TRANSMEMBRANE SERINE PROTEASE IN NEURAL PROGENITOR CELLS

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Recent studies show that the type II transmembrane serine proteases play important roles in diverse cellular activities and pathological processes through interaction with cell surface proteins, soluble proteins, proteins on adjacent cells and matrix components. Their expression and functions in central nervous system, however, are largely unexplored. In this study, we show that one such member, matriptase (MTP), is expressed in neural stem/progenitor (NS/P) cells and in neurons, but little or none in brain endothelial cells, astrocytes or microglial cells. MTP is crucial to NS/P cell mobility. Blocking of MTP expression or MTP activity impaired while overexpression of MTP promoted NS/P cell to traverse reconstituted basement membranes. Silencing of MTP impairs vascular endothelial growth factor and hepatocyte growth factor induced NS/P cell mobilization. MTP acts upstream of matrix metalloproteinases in promoting NS/P cell migration. In embryonic stem cell to neural differentiation, MTP has no effect on neural lineage entry by embryonic stem cell; high MTP protein or activity, however, shift the population between NS/P cells and neurons to favor neuronal differentiation. This is the first report to demonstrate the expression and function of type II transmembrane serine protease in NS/P cells

Poster Board Number: 3492

CONTRARY EFFECTS OF VALPROIC ACID ON THE DIFFERENTIATION OF STEM CELLS AT DIFFERENT STAGES OF DEVELOPMENT

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Valproic acid (VPA) is commonly used as a mood stabilizer and anticonvulsant. Despite the clinical relevance to neural disorders, the roles of this small molecule in neurogenesis are not well understood. The recent discovery of the inhibitory effect of VPA on histone deacetylase has led to studies on its potential application in manipulating differentiation of stem cells for therapy and regenerative medicine. We used an *in vitro* culture system to elucidate the biological effects of VPA on mouse embryonic stem (ES) cells D3 and E14Tg2a, primary neural stem (pNS) cells and neural precursor cell line C17.2. The morphological study in C17.2 differentiation suggested that seven-day treatment with VPA enhanced the neuronal development as evident of more extensive dendritic protrusions. On the contrary, embryoid bodies (EB) derived from VPA-treated ES cells for 14 days were reluctant to yield dendritic protrusions, as compared to the control group. Immunohistochemical staining demonstrated that the numbers (mean ± SD) of β-tubulin+ cells derived from both VPA-treated pNS cells and C17.2 cells were significantly higher than those of the control groups (pNS cells: VPA-treated vs. untreated; 13.2% ± 0.9% vs. 5.8% ± 0.6%; n=3; $p<0.01$, C17.2: VPA-treated vs. untreated; 37.8% ± 0.7% vs. 17.8% ± 0.6%; n=3; $p<0.01$), suggesting the differentiating effect of VPA on neural stem and progenitor cells. Besides, the numbers of 14-day VPA-treated D3 and E14Tg2a-derived EB with dendritic protrusions were comparatively lower than those of the untreated counterparts (D3: VPA-treated vs. untreated: 0% ± 0% vs. 32.6% ± 4.1%; n=3; $p<0.01$, E14Tg2a: VPA-treated vs. untreated: 0% ± 0% vs. 66.9% ± 5.1%; n=3; $p<0.01$), implying the inhibitory effect of VPA on the differentiation of ES cells. Using two-stage neural induction cultures, VPA treatment of D3 and E14Tg2a at an early stage (VN) elicited a significantly lower percentage of cell foci with β-tubulin+ protrusions compared to that with no treatment (NN, D3: VN vs. NN; 3.1% ± 0.8% vs. 32.6% ± 4.1%;

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n=3; p<0.01, E14Tg2a: VN vs. NN; 7.5% ± 4.3% vs. 66.9% ± 5.1%; VV vs. NV; 0% ± 0% vs. 92.0% ± 3.2%; n=3; p<0.01). On the contrary, VPA treatment at late stage (NV) yielded an elevated percentage of β-tubulin+ cell foci in E14Tg2a, implicating a late acting effect on neural commitment. Real-time PCR revealed two- to six-fold increases in the expression of Notch-1 and Notch-2, and the downstream targets, hes1 and hes3, in the VPA-treated ES cells E14. The up-regulation of the Notch signalling pathway might be attributable to the inhibitory effect of VPA on neural induction of ES cells. Taken together, the readouts imply the double-edged effect of VPA on the neural fate of stem cells at different stages of maturation.

Poster Board Number: 3494

MORPHOGENESIS, PATTERNING, AND DIFFERENTIATION OF ESC-DERIVED NEURAL ROSETTES

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Embryonic stem cell (ESC) *in vitro* neurogenesis resembles the development of the central nervous system in the early mammalian embryo. Using a well-defined monolayer protocol that utilizes the BMP-antagonist Noggin, we observe formation of neural rosettes, in which differentiating neural stem cells (NSCs) are radially arranged around a lumen and exhibit interkinetic nuclear migration. N-cadherin and Numb are localized at the apical luminal surface, suggesting the emergence of a polarized epithelial layer, reminiscent of radial glia in the embryonic neural tube. Rosettes also appear to have regional identities, for example expressing the ventral marker Nkx2.1 in one half of the rosette. In addition, our RT-PCR analysis shows that during differentiation, cells express markers of both dorsal (Pax7) and ventral neural populations (Mash1, Nkx2.1 and Dlx2). One goal of our work is to use this *in vitro* system to generate an enriched population of GABAergic interneurons that could be used to treat neurodegenerative diseases like Temporal Lobe Epilepsy (TLE), characterized by the loss of inhibitory GABAergic interneurons. We now demonstrate that further differentiation of hESC-NSCs efficiently produces mature GABAergic neurons.

Poster Board Number: 3496

LOCALIZED INDUCTION OF EARLY EMBRYONIC STEM CELL DIFFERENTIATION IN A NOVEL THREE DIMENSIONAL SYSTEM

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Embryonic stem cells (ESC) and induced pluripotent stem (iPS) cells are attractive cell sources for diverse clinical applications in regenerative medicine due to their ability to both undergo self renewal and to differentiate into specialized cell types of the adult body. Before the cell-based therapeutic potential of ESC can be implemented, a better understanding of the pathways regulating lineage-specific differentiation is required. This understanding will enable the development of better selective differentiation methods. Current studies suggest that similar pathways that are required for gastrulation and germ layer induction in the embryo are also essential for differentiation of ESC in culture. However, these pathways are still not fully understood. In order to investigate the mechanisms that control ESC differentiation and the cues that determine germ layer differentiation, a novel approach for controlled differentiation of ESC was developed. We formed embryoid bodies (EBs), from Brachyury:GFP mouse ES cells which serves as a kinetic mesodermal marker. Bmp-4 coated micro particles implanted in the EBs enable us to monitor the kinetics and localization of mesodermal differentiation by following GFP expressing cells proximal to the attached-beads along the differentiation process. In order to identify the early factors involved in ESC differentiation patterns by gene expression analysis, induced and non induced EBs were dissociated in to single cells and positive and negative populations of GFP were isolated using flow cytometry on various days of differentiation. Overall, our results demonstrate the ability to locally direct and control the differentiation of mouse ESC in a three dimensional system. Ongoing experiments are currently underway to further examine the differentiation process and the ability of different factors to locally induce or inhibit differentiation.

Poster Board Number: 3498

REGULATION OF ENDODERM COMMITMENT IN EMBRYONIC STEM CELLS BY FIBRILLAR FIBRONECTIN AND ADHESION STRENGTH

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Fibronectin (FN) is an extracellular matrix protein that assembles to a fibrillar network surrounding stem cells and significantly regulates morphogenesis, i.e. fibronectin null embryos are arrested at gastrulation. Here we also suggest that FN may also regulate embryonic stem cell (ESC) fate via its intrinsic properties, e.g. structure and stiffness. ESCs were found to have a spatiotemporal correlation between expression of FN and GATA4, an endoderm gene. Initial endoderm commitment depended on FN ligation of α5β1 integrins followed downstream by Src and Rho kinases, which was enhanced in ESCs cultured on stiff fibrillar FN matrix. To assess to what degree adhesion was required for this process, ESCs were exposed to high, radially-dependent shear force over a 6 day time course. The shear to detach 50% of the cells (τ50) did not change between 12 hr and 6 days, though it differed significantly between the matrices with different structure, i.e. τ50 of FN-coated substrates was 5-fold higher than fibrillar FN matrix. The formation and uniform distribution of small focal contacts observed throughout cells on fibrillar FN was found to correlate with higher adhesion strength and endoderm differentiation, e.g. the ratio of GATA4 positive to negative cells in areas of high shear forces increased ~50 times. Together these data suggest that the matrix ligand, structure, and stiffness are all important in endoderm commitment, and that a uniform distribution of labile adhesions is required to maintain firm adhesion characteristic of endoderm.

Poster Board Number: 3500

A NOVEL ROLE FOR ANKRD11 IN THE DEVELOPING CEREBRAL CORTEX

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Increasing evidence suggests that perturbations in cell genesis during the development of the nervous system may underlie cognitive dysfunction associated with neurodevelopmental disorders. In that regard, we have identified a novel role for a recently identified autism-associated gene, ankyrin repeat domain-containing protein 11 (Ankrd11), in the development of the cerebral cortex. We show that Ankrd11 is expressed in both embryonic cortical precursors and in newly-born cortical neurons. We demonstrate that genetic knockdown of Ankrd11 inhibits neurogenesis in cultured cortical precursors. Embryonic neurogenesis was similarly perturbed after acute shRNA-mediated knockdown of Ankrd11 *in vivo* using *in utero* electroporation. Furthermore, we have developed a method for generating neural precursors from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), based on inhibition of BMP signalling. Precursors generated using this method express markers consistent with a forebrain precursor identity and subsequently differentiate into glutamatergic neurons. We have employed this method to study the role of Ankrd11 in human neurogenesis.

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Poster Board Number: 3502

MASS TRANSFER LIMITATIONS IN EMBRYONIC STEM CELL DERIVED EMBRYOID BODIES DURING DIFFERENTIATION

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Human embryonic stem cells (ESCs) readily proliferate, self-renew and can be driven to differentiate into cell types from all three germ layers making them a source with tremendous potential in the treatment of debilitating diseases and injuries. Typically *in vitro* differentiation of these cells is induced via embryoid body formation to mimic *in vivo* conditions of embryo development. However, the use of hESC-derived cells in clinical applications will require culture protocols which are well defined, in a controlled environment, and fully reproducible to successfully scale up production to clinically relevant numbers. The ability to accurately direct the differentiation of these cells is required to minimize the risk of tumour formation. As such, we have developed a transient mass diffusion model to elucidate the effect of nutrient transfer and embryoid body size on subsequent cell differentiation. The results of this model indicate that oxygen levels at the centre of large embryoid (400μm) bodies are significantly lower than in small embryoid bodies (200μm) which helps substantiate experimental observations by others indicating that embryoid body size influences differentiation trajectories. The glucose concentration profile was modeled and resulted in a profile which did not depend on embryoid body size. As such, glucose concentration was not shown to be a major contributor to hESC differentiation. A comparison of the model results with published experimental data reveals a correlation between the fraction of cells exposed to different oxygen and cytokine concentrations and the fraction of cells observed to be differentiating into a specific lineage. A better understanding of diffusive mass transfer within the embryoid body is an important step in the development of robust differentiation protocols required to produce functioning cell types for tissue engineering applications. The model was validated against experimental data with from mouse and human ESCs.

Poster Board Number: 3504

ASTROCYTIC DIFFERENTIATION OF EMBRYONIC STEM CELL-DERIVED OLIG2 POSITIVE GLIAL PROGENITOR CELLS

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Astrocytes are the major glial cells in the central nervous system (CNS). Previous studies have demonstrated that brain-derived immature but not mature astrocytes have the ability to promote axon growth. Moreover, transplanted immature astrocytes are motile, interact with blood vessels and suppress glial scar formation, while transplanted mature astrocytes do not. Given these potential beneficial effects, immature astrocytes may be used as cell therapy for CNS injury or diseases. Embryonic stem cells (ESCs) are capable of self-renewal and can give rise to neural lineage cells, proving the promise of using ESC-derivates as cell replacement therapies for CNS disorders. To date, very few studies have examined astrocyte differentiation from ESCs. Astrocyte differentiation has often been reported merely as a by-product in directed differentiation of ESCs into neurons or oligodendrocytes. The Olig2-GFP human (h) and mouse (m) ESCs are genetically labeled ES reporter cell lines, in which GFP is inserted into the Olig2 gene locus so that ES-derived cells that express Olig2 also express GFP. Our previous studies have shown that using small molecule-based methods (Jiang et al. 2010 and Xue et al., 2009), both Olig2-hESCs and Olig2-mESCs can be coaxed into either Olig2+/GFP+ motor neuron precursors or glial progenitor cells (GPCs) that can then give rise to oligodendrocytes. Here, we studied the fate of the Olig2+/GFP+ GPCs cultured in the presence of the astrogliogenesis inducer bone morphogenetic protein-4 (BMP-4). Although the GFP expression was terminated once the ESC-derivates stopped expressing Olig2, the cells that once expressed GFP protein could be identified using anti-GFP antibody even 5 days after culturing in the presence of BMP-4. Thus, we used anti-GFP staining to track down the fate of Olig2+/GFP+ cells after exposure to BMP-4. Interestingly, the mESC-derived Olig2+/GFP+ GPCs could be efficiently differentiated into GFAP+ astrocytes in the presence of BMP-4. The majority of the differentiated cells was GFAP+/A2B5- type-1 astrocytes and a small population of them was GFAP+/A2B5+ type-2 astrocytes. We are studying whether these ESC-derived astrocytes have similar properties of brain-derived immature astrocytes to promote neurite growth when co-cultured with brain- or ESC-derived neurons *in vitro* and to promote axon outgrowth and suppress scar formation in CNS lesions after transplantation *in vivo*.

Poster Board Number: 3506

COMPARATIVE ANALYSIS OF BRANCHING EPITHELIUM DERIVED FROM EMBRYONIC PDX1+ PANCREAS AND DIFFERENTIATED EMBRYONIC STEM CELLS

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We have generated an embryonic stem cell (ESC) line in which sequences encoding green fluorescent protein (GFP) were targeted to the locus of the pancreatic-duodenal homeobox gene (Pdx1). Using the reporter Pdx1GFP/w ESC line, we have utilised a serum-free based approach to induce the formation of Pdx1+ endoderm through the sequential addition of BMP4 (5 ng/ml) from day (d) 0 to d4, and subsequently a pulse of retinoic acid from d4 to d5. Under these conditions, GFP+ cells formed well-defined tube-like structures (termed GFPdull structures) within d12 embryoid bodies (EBs) that were connected to GFP bright (GFPbr) buds. These GFPbr cells from partially disaggregated d12 EBs, formed epithelial ring-like structures when cultured in matrigel. After several days in culture, these rings undergo a process resembling branching morphogenesis, similar to that seen during pancreatic development of the mid-gestation embryo. Indeed, when pancreatic buds from E12.5-E13.5 Pdx1GFP/w embryos were dissociated and cultured under identical conditions, a virtually indistinguishable process of proliferation and branching morphogenesis of epithelial rings was observed. Furthermore, immunohistochemical analysis of both embryonic Pdx1GFP/w branching pancreatic epithelium and d12 GFPbr branching organoids, revealed that they expressed a suite of markers that were representative of epithelial (EpCAM, E-Cadherin), ductal (MUC1), exocrine (Amylase and Carboxypeptidase 1A) and endocrine cell types (Glucagon, Somatostatin). In summary, ESCs can be directed to differentiate into pancreatic endoderm *in vitro*. It is hoped that these studies will assist in the understanding of pancreatic cell fate in the mammalian embryo, and further efforts towards discovering therapeutic applications in the treatment of Type 1 Diabetes.

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EMBRYONIC STEM CELL PLURIPOTENCY

Poster Board Number: 3510

OXYGEN-REGULATED TRANSCRIPTIONAL NETWORKS CONTROLLING HUMAN EMBRYONIC STEM CELL PLURIPOTENCY

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The pluripotent human embryonic stem cells (hESC) are isolated from the inner cell mass (ICM) of blastocyst stage embryos. These embryos receive metabolites and oxygen from the uterine fluid containing 2% - 5% of dissolved oxygen. Early embryo develops in low oxygen environment suggesting that hypoxic growth conditions would be physiological for hESC cultures. Indeed, hypoxic growth conditions (1% - 4% O2) reduce spontaneous differentiation and enhance hESC self-renewal indicating that the processes mediating spontaneous differentiation are suppressed under low oxygen. However, how hypoxia contributes to pluripotency and self-renewal is not completely understood. To study the mechanisms involved in oxygen-regulated pluripotency and self-renewal, three different hES cell lines (HS401, H9 and HS360) were plated on Matrigel® and cultured in 4% (hypoxia) or in ambient oxygen concentration for fixed time intervals. Consistent with the hypothesis of hypoxia supporting pluripotency, the data from Western and Flow Cytometry analyses revealed that in response to low oxygen environment, hES cells activate canonical hypoxia responses and inhibit the down regulation of the pluripotency markers. To identify the hES cell specific transcriptional programs regulated by oxygen, RNA was extracted from all the time points for which microarray analysis, using the Affymetrix Human Exon 1.0 ST array platform. Data analysis revealed that hypoxic culture induced differential expression of over 200 genes, affecting various biological processes regulating hESC pluripotency and differentiation.

Poster Board Number: 3512

DEVELOPMENT OF A DEFINED SERUM FREE AND XENO FREE MEDIUM COMPOSED OF MINIMAL COMPONENTS FOR CULTURING HUMAN ES/IPS CELLS

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Previously, we developed a serum-free growth factor defined culture medium, hESF9, for culturing human ES cells without feeders. However, it contained non-human origin components. Here we report a modified version of hESF9 that is completely xeno-free and composed of minimal components. Many other xeno-free media contain large quantity of albumin and growth factor cocktail to aid the maintenance and proliferation of pluripotent cells, but at the same time, they mask minute reactions of cells. On the contrary, this xeno-free culture medium is composed of only 3 growth factors and it is with the lowest quantity of human albumin. Thus cells cultured in this medium would yield more sensitive reaction to signals caused by chemical compounds of interest. So the use of this media in research hold promise for establishment of not only safe clinical application but also assured drug screening. Using this medium, we performed long-term (>10 passages) culture of human ES/iPS cells and confirmed their maintenance of an undifferentiated state. Human ES/iPS cells cultured in this medium maintained a normal karyotype and expression of pluripotency markers which were assayed by FACS, PCR and immunofluorescence analysis. Furthermore, we confirmed multilineage differentiation potential of these cells by the embryoid body assay.

Poster Board Number: 3514

ACELLULAR XENO-FREE LAMININ 511 RICH CULTURE MATRIX SUPPORTS HUMAN EMBRYONIC STEM CELLS

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Several recent papers, including our own, have illustrated that laminin 511 (Lm-511) coating alone supports maintenance of undifferentiated human pluripotent stem cells (hPSC) in defined culture conditions. Therefore, either purified or recombinant Lm-511 seems to be an optimal extracellular matrix coating for hPSCs. However, the difficulty and high cost of purification or recombinant production of Lm-511 limits its use in large-scale use. We have taken an alternative approach in order to develop an optimal and cost-effective human derived culture matrix for the hPSC cultures. This is based on the use of an acellular culture matrix (AMX) derived by alkaline lysis of a human chorioncarcinoma cell line that produces large amounts of laminins -511 and -111, the only two laminin isoforms also synthesized by hESCs. The hESC line FES 29 was passaged on AMX coated plates in defined medium (StemPro). Flow cytometry analysis after 15 passages showed that 86% and 87% of the cells cultured on AMX were positive for the surface markers SSEA3 and Tra 1-60, respectively, while the corresponding numbers for the control cells cultured on Matrigel were 89% and 91%. The AMX-cultured cells also exhibited a strong reactivity for Nanog, Oct4 and E-cadherin antigens in immunocytochemistry. The cells differentiated into all three germ layers in embryoid body assay. This acellular matrix has many advantages. It is derived from human cells and it is easy and cheap to prepare as compared with most commercial matrices. Furthermore, there is no batch to batch variation when equal numbers of cells are plated each time when preparing the plates. We assume that out of the two laminin isoforms produced by the chorioncarcinoma cell line, Lm-511 is the one supporting the undifferentiated hPSCs. To demonstrate it we decided to knock down the laminin alpha 5 subunit in the chorioncarcinoma cells and to prepare culture matrices that contain no laminin alpha-5 protein. We're currently proceeding with these and several other strategies to investigate the role of Lm-511 in the biology of hPSCs.

Poster Board Number: 3516

INVESTIGATING THE EFFECTS OF EXTRACELLULAR MATRIX COMPONENTS ON THE REGULATION OF PLURIPOTENCY

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Human embryonic stem cells are pluripotent cells that have indefinite replicative potential and ability to differentiate into derivatives of all three germ layers. HESCs are conventionally grown on mitotically inactivated mouse embryonic fibroblasts and some alternative feeder types of human origin have been used to culture hESCs while trying to prevent cross-species contamination. Understanding the mechanisms that drive these cells to proliferate and specialize into certain lineages is important to our understanding of pluripotency and for controlling their behaviours *in vitro*. The trophic factors that are secreted by the feeders have been found to be important for long-term pluripotency of hESCs. However, there are also supportive culture systems for hESCs lacking feeder cells and in these the substrate has been shown to be important, suggesting that extracellular matrix-stem cell interactions might contribute to the regulation of self-renewal or differen-

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tiation. Extracellular matrix components are known to form an important component of the microenvironment of the niche for other types of stem cells but have been relatively little investigated for ESCs. Therefore we used mass spectrometry for analyzing the niche of hES cell lines grown on different feeder cells, to reveal the functional components of the extracellular matrix which might take part in the regulation of self-renewal. The aim of our study is to investigate ECM components which might have a role in the maintenance of hESCs by comparing matrices from human placental stromal fibroblasts and mouse embryonic fibroblasts. hPSFs were shown to support long-term pluripotency of hESC for at least 12 passages and the matrix laid down by immortalized placental fibroblasts was found to be supportive for hESC growth and pluripotency in the absence of the cell layer. We used a proteomics approach to identify ECM proteins released by mouse and human feeders in order to characterise the range of extracellular matrix components that support the growth of self-renewing hESCs. We aimed to determine both similarities and differences between supportive and unsupportive feeder and so to dissect important and novel components of the matrix, which conducive with the pluripotent self-renewing state. We found that many ECM proteins are expressed by both mouse and human feeders. However some candidate proteins are only expressed by supportive matrices and might play a role in the maintenance of pluripotency. Quantitative differences were also identified between supportive and unsupportive matrices. We tested candidate extracellular matrix molecules as substrates for feeder-free growth of hESCs including HSPG, fibulin-2, collagen VI and tenascin C. Our results show that some of the molecules can support attachment and self-renewal of hESCs alone or in combination with fibronectin in the absence of feeders. Thus this study further illuminates the role that ECM interactions play in the hESCs phenotype which has until recently been a neglected area of hESCs biology.

Poster Board Number: 3518

THE CORNING® SYNTHEMAX™ SURFACE: A SYNTHETIC, XENO-FREE SURFACE FOR LONG-TERM SELF-RENEWAL OF HUMAN EMBRYONIC STEM CELLS IN DEFINED MEDIA.

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Scalable, reproducible, low cost and regulatory-friendly technologies must be developed to enable clinical use of human embryonic stem cell (hESC)-based therapeutics. hESC culture methods use complex, animal-derived products, such as mouse feeder layers, Matrigel™, murine laminin or human-derived biological substances as surfaces to which the hESCs attach. Most of these materials are costly, of limited scalability, have batch to batch variability and are a potential source of adventitious agents. For clinical application of hESC-based therapeutics it is highly desirable to have defined, scalable culture systems for production of cells suitable for clinical use. In this study we describe a fully synthetic, xeno-free surface for the culture of undifferentiated hESC. A peptide sequence derived from the active domain of the vitronectin protein was covalently linked to a synthetic acrylate polymer surface to mimic biological ligands for cell adhesion. Self-renewal and pluripotency of multiple hESC lines (H7, H1, H9, and BG01v) cultured on Synthemax Surface was compared to cells grown on Matrigel control surfaces under various defined media conditions (StemPro®, NutriStem™, mTeSR®1, and XVIVO™ 10). Our results demonstrate efficient adhesion and self-renewal of H7, H1, H9 and BG01v/hOG hESCs on Synthemax Surface for up to 20 serial passages in defined media. Importantly, stable proliferation rate, expression of stem cell specific markers (Oct-4, TRA 1-60, SSEA-4), *in vitro* and *in vivo* pluripotency and normal karyotype were retained throughout multiple passages on Synthemax Surface. Further, we demonstrated successful scale-up of Synthemax Surface to large culture vessel formats to accommodate the clinical scale production of therapeutic cells. To our knowledge, Synthemax Surface is the only commercially available, synthetic, non-biological surface that supports the long-term, multi-passage expansion of undifferentiated hESC in chemically-defined, xeno-free media. We believe Synthemax Surface will be applicable for both research purposes and scalable manufacturing of hESC-derived cellular therapeutics.

Poster Board Number: 3520

THERMALLY-RESPONSIVE SYNTHETIC POLYMER HYDROGELS FOR ENZYME FREE PASSAGING AND LONG TERM MAINTENANCE OF HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cell culture environments have generally relied on provision of recombinant or purified extracellular protein matrices to support attachment and growth. Here we report the screening of synthetic polymer hydrogels as a chemically defined and protein-free alternative to purified or recombinant extracellular matrix molecules for hESC attachment, maintenance and enzyme free passaging. Specifically we report the identification of two hydrogel polymers which maintain undifferentiated hESC marker and endoderm, mesoderm and ectoderm lineage potential in a defined medium, mTESR, for over 20 passages in the absence of enzyme-mediated passaging, a capacity confirmed in independent cell lines. These substrates constitute a paradigm shift in stem cell culture technology, obviating reliance on both animal sourced or recombinant protein substrates and enzymatic dissociation for passaging.

Poster Board Number: 3522

LOCALIZATION AND FUNCTION OF SURVIVIN SPLICE VARIANTS IN HUMAN EMBRYONIC STEM CELLS

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The BIRC5 gene encodes the dual function protein Survivin as well as four additional splice variants (Survivin, Survivin ΔEx3, Survivin 2B, Survivin 3B and Survivin 2α). Previous results from our lab and others have demonstrated that Survivin is expressed at high levels in embryonic stem cells and that inhibition of Survivin function in these cells results in apoptosis, however these previous studies did not investigate the other four splice variants. In this study, we examined the expression, subcellular localization and function of the other variants. Consistent with previous results studying Survivin, all five splice variants are expressed in human embryonic stem (hES) cells and all five variants were expressed at levels significantly higher than both hES derived differentiated cells and somatic cells. Survivin and Survivin ΔEx3 are the two most common variants expressed, accounting for 90% of the expression from the Birc5 gene. Survivin functions as both a necessary member of the chromosomal passenger protein complex and as a member of the inhibitor of apoptosis (IAP) family based on its' single baculovirus IAP repeat. The functions of the other four variants are not well understood but a very small number of studies suggest they may be important in differentiation. For this reason, we quantitated mRNA expression following differentiation with retinoic acid. Differentiation resulted in a rapid reduction

of all expression levels over a short timecourse but did not demonstrably change the ratio of splice variants to each other. We also examined the subcellular localization of three of the variants. Survivin displayed canonical chromosomal passenger protein (CPP) localization to the centromeres during prophase, moving to the inner centromeric region during metaphase and onto the interzonal microtubules at telophase. During interphase it was cytoplasmic due to a nuclear export signal. In contrast, Survivin ΔEx3 cannot function as a CPP and has no well defined function. It has a bipartite nuclear localization signal and was found constitutively in the nucleus. When we examined Survivin 2B localization we found that this variant also does not localize as a CPP and instead was distributed along the chromosomes during mitosis and also to the mitotic spindle poles. These disparate localizations suggest alternative functions for the splice variants from those commonly described for Survivin. Previous studies have demonstrated that inhibition of Survivin function in hES cells through siRNA or expression of a dominant negative mutant resulted in increased cell death and reduction in the ability to form teratomas. We sought to repeat these studies with an inducible shRNA system to elucidate any pleiotropic effects of Survivin inhibition masked by the apoptosis caused by unregulated inhibition. Reduction of Survivin and Survivin ΔEx3 mRNA expression by approx. 50% resulted in a corresponding reduction in Oct-4 expression. Together these results have led to the hypothesis that Survivin has functions in embryonic stem cells in addition to those canonically described and these functions may be dependent on the splice variants. Additionally, embryonic stem cells are the perfect cell type for examining Survivin function as all variants are expressed at detectable levels unlike most cell types and ES cells can be differentiated into multiple lineages to determine changes in splice variant expression and function during development.

Poster Board Number: 3524

SIMULTANEOUS SCREENING OF HUMAN PLURIPOTENT STEM CELL, NEURECTODERM, AND PRIMITIVE STREAK SUBPOPULATIONS IN MICROENVIRONMENTS ENGINEERED FOR ROBUST RESPONSIVENESS

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With the advent of human pluripotent stem cells (hPSCs) there has been a great interest in understanding the regulation of stem cell fate in order to further our knowledge of human development and to enable drug development and cell therapy. Currently, assays do not account for the complex microenvironments, or "niches", in which the hPSCs reside. As in the embryo, these niches have heterogeneous spatial organization and multiple populations of factor secreting cells. These microenvironmental heterogeneities confound results, reduce assay robustness, and limit our ability to gain mechanistic insight into stem cell fate processes. Micro-contact printing of hPSCs into colonies of specified shape, size, and colony-to-colony separation distance can be used to engineer the microenvironment and mitigate the heterogeneous response and obfuscating endogenous signalling in hPSC culture. We have developed a robust and scalable micro-contact printing based HTP (μCP-HTP) platform, consisting of hPSC colonies arrayed in 96-well plates and we have optimized colony size, colony spacing, cell density, media composition, and the substrate for optimal signal to noise ratio. Single cell protein expression profiling of 20+ transcription factors and cell surface receptors across a diverse set of conditions revealed rapid induction of neurectoderm and primitive streak/mesendoderm lineages within 48 hours, in addition to maintenance of pluripotency. Using hierarchical clustering of these protein expression profiles, we determined dual staining of Oct4 and Sox2 can allow discrimination between pluripotent, neurectoderm, extraembryonic, and primitive streak subpopulations, allowing the simultaneous tracking of these subpopulations as screen readouts. We have completed an initial screen using the human embryonic stem cell (hESC) H9 cell line, obtaining results which link 300+ combinations of ligands and their small molecules inhibitors to hESC fate. We have elucidated the mechanistic role of individual signalling factors in stem cell fate decisions, identifying synergistic and antagonistic relationships between factors, and developed optimized conditions for both stem cell expansion and directed differentiation. Dose curves of single factors reveal a common bimodal response of stem cells to ligands, where subpopulations have dramatically different responses to ligands at different concentrations, particularly FGF2, Activin A, and BMP4, necessitating careful dosing of ligands. Several small molecules have been identified that induce neurectoderm and primitive streak. The dual gain and loss of function screen design has also enabled functional assessment of endogenous signalling, indicating generally low levels of most endogenous signalling in this platform with the exception of high intracrine FGF2. Lastly, the μCP-HTP platform will enable the generation of quantitative and mechanistic systems-level models of fate decisions in specific hPSC cell lines and their derivatives, allowing cell-line comparative studies and step-wise optimization of differentiation.

Poster Board Number: 3526

SECONDARY NETWORKS MODULATE HUMAN PLURIPOTENT STEM CELL DIVERSITY

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Pluripotency is a transient developmental state in the early embryo defined by the capacity to generate all cell types comprising an adult organism. In humans, this unique developmental potential can be stabilized through establishment of embryonic stem cell (ESC) lines and can be induced in somatic cells (induced pluripotent stem cells; iPSCs) via transcription factor mediated epigenetic reprogramming or fusion with ESCs. The unique combination of unlimited growth and an extensive differentiation repertoire makes human pluripotent stem cells (hPSCs) attractive tools for modeling human developmental and degenerative processes. However, these studies have been complicated by an indisputable heterogeneity in the behavior of hPSCs. Further, that pluripotency can be both readily acquired via epigenetic reprogramming and silenced via differentiation demonstrates the context-dependent dominance of pluripotency networks. Collectively, these observations highlight the complexity of pluripotency networks and necessitate a more thorough understanding of the underlying dynamics. To this end, we developed a systems biology approach based on genome-wide SNP, mRNA, miRNA and DNA methylation data from more than 300 hPSC samples and 200 somatic cell lines and tissue samples. We find that while the hPSCs are all clearly distinct from differentiated cells, a broad spectrum of differing molecular profiles exists among them. Analysis of the expression profiles showed that all hPSCs share invariant pluripotency-associated molecular networks, and indicated that secondary networks underlie the variation in molecular profiles. Our results show that pluripotency has tolerance for considerable variation within the distinct boundaries that separate all pluripotent cells from all non-pluripotent cells. We are investigating how pluripotent sub-states are maintained and why some specification factors are tolerated in hPSCs while others are silenced in iPSC generation.

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Poster Board Number: 3528

CHARACTERIZATION OF HUMAN EMBRYONIC STEM CELL PATHWAYS AT SUBCELLULAR RESOLUTION

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Properties of human embryonic stem cells (hESCs) such as pluripotency and differentiation are regulated by a complex network of interacting pathways. The subcellular localization of these pathway proteins and their relative abundance within a pathway dictate the influence of the pathway on hESC fate.. Further, the pool of endogeneously secreted extracellular cytokines also dictates activation/repression of various pathways. Here we describe an effort to uncover these regulatory mechanisms in hESCs through comprehensive characterization of the hESC proteome at subcellular resolution. We developed a discontinuous sucrose gradient-based protocol for simultaneous isolation of the nuclear, membrane and cytosolic subcellular fractions for studying differential localization of proteins inside the cell, and of the endoplasmic reticulum (ER) to study localization of proteins in the extracellular space (the secretome); secreted proteins are synthesized in the (ER). Further, we also used the method of spectral counting to estimate the relative abundance of all proteins identified in the cytosolic and ER fractions. The relative abundance data sheds light on dominant mechanisms regulating a particular pathway. The integration of protein identifications with subcellular localization data and estimates of protein abundance allowed us to uncover several potential mechanisms pertaining regulating hESC pluripotency and differentiation. We first studied the pluripotent hESC state and identified several chromatin modifying proteins to be experimentally sequestered in the cytosol. Our data suggests that the activity of the SIN3A chromatin remodeling complex and several p300/CBP containing transcription factor complexes may be regulated through cytosolic sequestration. Several negative regulators of the SMAD2/3 pathway are expressed in the cytosol, suggesting that the SMAD2/3 pathway is highly regulated in undifferentiated hESCs. We also studied the secretome of hESCs grown in conditioned medium (CM), to elucidate the endogenous signaling present in the hESC microenvironment. Relative quantification for these secreted proteins allows us to identify the dominant signaling pathways present, as well as mechanisms of their regulation. We coupled this data with relative quantification of mouse embryonic fibroblast (MEF)-secreted proteins present in CM, to understand the regulation of endogenous signaling pathways by exogenous MEF factors in CM. Finally, we studied the differentiation of hESCs towards trophectodermal lineages by inhibition of Activin/Nodal pathway and identified various regulatory feedback loops that control SMAD signaling when differentiation is triggered. Taken together, our data enables us to obtain a comprehensive understanding of the signaling networks operational in hESCs and the mechanisms that regulate them, at subcellular resolution.

Poster Board Number: 3530

EVALUATION OF PROSPECTIVELY SORTED HUMAN EMBRYONIC STEM CELL-DERIVED MULTIPOTENT NEURAL STEM CELLS FOR TUMORIGENESIS AND NEURAL LINEAGE RESTRICTION

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Multipotent neural stem cells (NSC) derived from human embryonic stem (hES) cells, fetal/adult tissues, or reprogrammed somatic cells (induced pluriopotent stem cells, hiPS) can differentiate into all central nervous system CNS) cell types. As we have shown, human fetal NSC prospectively sorted for a CD133+/CD34- population differentiated primarily into oligodendrocytes and neurons, and promoted functional recovery when transplanted 9 or 30 days post-spinal cord injury (SCI) (PMID: PMC1216836 & PMC2923623); these cells exhibited no evidence of tumorigenesis *in vivo*. However, the properties of hNSC derived from hES and hiPS cells, including their fate and tumorigenesis potential, and potential for efficacy in the treatment of neurological disease/injury are unclear, particularly given that hES and hiPS exhibit high levels of heterogeneity between lines and following differentiation. Experiments with hESC have shown that selection for CD133+ cells yields a population of neuroectoderm and differentiation committed cells, suggesting that not all cells in hESC cultures are pluripotent. We hypothesize that the differentiation of pluripotent hESC or hiPSC to multipotent NSC, followed by selection via magnetic bead activated cell (MAC) sorting to select for CD133+ cells, will yield a population of fully neural lineage restricted NSC, reducing the tumorigenesis potential of the pluripotent starter population. Additional selection for CD34- cells is hypothesized to limit hematopoietic cells in culture and further reduce heterogeneity. Here, we demonstrate that this sorting protocol can efficiently select a sustainable hES-derived NSC population (~96.4% CD133+) as assessed by flow cytometric analysis after 4 passages (P4) post-sorting, compared to the unsorted cell population (~50.0% CD133+). NSC purity was sustained up to P12 (~92.7% CD133+). Furthermore, sorted NSC had significantly less hematopoietic contaminants (CD133+/CD34+) than unsorted NSC at P4. However, this CD133+/CD34+ population increased slightly after P11, suggesting that negative selection for hematopoietic contaminants may not be sustained at passages greater than P11. Preliminary fate analysis after 10DIV under differentiating conditions showed that both cell populations (unsorted and sorted) had minimal GFAP+ cell number, while sorted cells had a significant (~50%) reduction of β-tubulin III+ cells. Furthermore, sorted cell populations expressed markers suggestive of early neuronal development that included nestin, β-tubulin III, and the radial glial marker Brain Lipid Binding Protein (BLBP). Preliminary data for cell proliferation was assessed by EdU incorporation as well as q-PCR, showing low to no difference in the proliferation rate between sorted and unsorted populations, suggesting low tumorigenesis potential in both cases. Currently, we are validating this MAC sorting protocol using other hES-NSC and hiPS-NSC lines, and characterizing their neural developmental stage and lineage potentials. Teratoma formation assays are in progress to characterize the effect of CD133+/CD34- sorting on tumorigenesis potential. These data may have implications for the generation of hES and iPS-derived NSC lines with improved safety and efficacy profiles for pre-clinical testing in neurological disease/injury indications.

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Poster Board Number: 3532

HUMAN EMBRYONIC STEM CELLS AND FLOW CYTOMETRY: CONSIDERATIONS FOR CHOOSING RIGHT CONTROLS

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In vitro culturing of human embryonic stem cells (hESC) is a promising tool to explore the possibilities of using pluripotent cells for future medicine. The characterization and maintenance of cell pluripotency is of utmost importance during cultivation of cells and performing experiments. The heterogeneity of hESC culture due to spontaneous differentiation is a well-known fact, which can only be quantified at the level of single cell analysis. Here we used multicolor flow cytometry to detect simultaneously up to three pluripotency marker proteins in individual human embryonic stem cells. Fluorescence minus one (FMO) controls are widely suggested to use for multicolor experiments. We suggest to use an alternative control, where the specific isotype control antibody is added to the FMO control sample (FMOi). This type of control should be used in cases, when non-specific and specific antibody stainings show partially overlapping fluorescence signals. Nonpluripotent cells present within a pluripotent cell population can be also used as a negative control for antibodies to detect markers for pluripotency. In addition we also suggest an alternative gating approach to combine fluorescence intensities for a cell cycle marker (DNA stained with DAPI) and negative control for pluripotency marker on density-plot graphs. This enabled us to show, how nonspecific staining and autofluorescence increase as the cell cycle proceeds. To clarify the result, we used an "aslant" gating system for detecting antibody reaction adequately without counting possible false events in G1 or G2/M phases. We conclude that nonspecific binding should be carefully and critically controlled, when immunostaining of human embryonic stem cells is carried out.

Poster Board Number: 3534

RECOMBINANT VITRONECTIN SIGNALLING PATTERNS IN HUMAN EMBRYONIC STEM CELLS

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The extracellular matrix (ECM) prote*in Vitro*nectin is a promising candidate for creation of defined surfaces that support human embryonic stem cell (hESC) growth and maintenance. Vitronectin is a 75kDa ECM molecule that promotes cell spreading and adhesion through interactions with cell surface integrins. We have recently shown that hESC lines MEL1, MEL2 and HES3 can be supported for greater than 10 passages on tissue culture plastic surfaces coated with a recombinantly produced VN fragment consisting of the somatomedin B domain and integrin associating RGD motif. HESC support on the VN fragment was equivalent in pluripotency marker expression, proliferation and differentiation potential compared to controls grown on the current ECM standard derived from an Engel-Breth-Holm Swarm mouse tumour (GeltrexTM). VN, through interactions with its known integrin binding partners (αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, αIIbβ3) is known to influence differentiation, proliferation and cell survival in a variety of different cell types however the downstream signaling pathways of VN in hESC have not yet been elucidated. For this paper, we initially screened changes in intracellular phosphorylated protein signaling between hESC grown on a recombinant VN fragment versus the less defined current standard GeltrexTM (Invitrogen). It was discovered in this initial screen that phosphorylation of tumour suppressor protein p53, was down regulated at three different locations, S15, S46 and S392 in cultures grown on VN. Here we report some preliminary investigations into changes in p53 phosphorylation on VN and the concomitant effects on cell survival and pluripotency. This research will have significant impact on optimization of defined culture processes resulting in effective scale-up of hESC for therapeutic applications.

Poster Board Number: 3536

EFFECTS OF DGCR8 KNOCKDOWN ON HUMAN EMBRYONIC AND ADULT STEM CELLS

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Small noncoding RNA molecules have emerged as a key post-translational regulator of gene expression in diverse species. In particular, microRNAs have been demonstrated to play an integral role as regulators of stem cell pluripotency, proliferation and differentiation. Novel technologies such as deep sequencing have enabled identification of 500-1000 novel microRNAs that may have regulatory roles in stem cell function. In mouse cells, mutant ESC lacking microRNAs have been utilized to link microRNAs with specific cellular responses. In this study we utilize a similar approach to create knockdown of DGCR8, a key component in the microRNA biosynthesis pathway. The DGCR8 RNAi with a GFP cassette to aid visualization of transfected/transduced cells was developed. Two gene delivery platforms were utilized: BacMam, an insect-based nonintegrating viral method known to achieve efficient gene delivery for transient expression into hard-to-transfect primary and stem cells and episomal vectors, EBV-based nonintegrating vectors that aids in rapid creation of pooled stable clones. DGCR8 RNAi was tested in Ntera2 human embryonal carcinoma cells, human embryonic stem cells (hESC) and human adipose derived mesenchymal stem cells (AdSC). Preliminary results indicated a decrease in endogenous levels of the DGCR8 level with RNAi expression in all the cells types. In the case of hESC and AdSC, distinct cellular phenotypes were noted in DGCR8 RNAi treated cells relative to control. Further analysis of global gene expression analysis together with miRNA expression patterns will allow confirmation of large numbers of microRNAs while linking the role of microRNAs to specific processes involved in stem cell maintenance and differentiation.

Poster Board Number: 3538

IDENTIFICATION OF MARKER GENES FOR THE EARLY DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

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Several markers for human embryonic stem cells (hESCs) have been identified. However, the function and molecular mechanisms underlying the unique properties of hESC are still poorly understood. The aim of our study is to elucidate the regulation of self-renewal and early differentiation of hESCs. For this purpose we have analyzed unpublished and published transcriptome data on hESCs and their differentiated derivatives from altogether 21 hESC lines. From this data we have identified the genes rapidly down-regulated or induced during the early differentiation of different hESC lines.

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Two novel candidate genes have been chosen for further functional studies to examine their importance in the hESC pluripotency maintenance.

Poster Board Number: 3540

HUMAN RECOMBINANT LAMININ-521 COATING PERMITS SURVIVAL OF DISSOCIATED PLURIPOTENT HUMAN STEM CELLS INTO SINGLE CELL SUSPENSION AND SUPPORTS THEIR LONG-TERM SELF-RENEWAL IN XENO-FREE AND DEFINED ENVIRONMENT

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We have recently shown that laminin-511 alone can support long-term self-renewal of pluripotent human embryonic and iPS (hES/iPS) cells in a xeno-free cell culture environment. However, laminin-511 failed to permit stable survival of the cells after replating from single cell suspension. In the present study, we have produced recombinant human laminin-521, another laminin isoform expressed by pluripotent hES cells and a part of their natural niche. Laminin-521 was also shown to support self-renewal of hES cells in a completely defined, feeder-free and xeno-free cell culture system using TeSR2TM medium. Importantly, however, this laminin isoform allowed clonal survival of pluripotent hES cells after plating from single cell suspension, and subsequent long-term self-renewal at a stable rate higher than that of cells grown on Matrigel. The cells were passed every 10-12 days in 1:20-1:30 ratios, cultured as monolayers for over 4 months exhibiting stable expression levels of pluripotency markers (Oct-4 and Nanog), after which the cells could develop teratomas in nude mice, containing cell lineages of all three germ layers. Pluripotency of the cells was also confirmed by embryoid body formation with subsequent immunofluorescence study. The pluripotent cell clonal survival was mediated via interaction with alpha6/beta1 integrin. This new chemically defined and xeno-free human pluripotent stem cell culture system may be particularly useful for producing large quantity of human stem cells for cell therapy. The method is also advantageous both timewise and laborwise, it is cost-effective and can be automated, and, thus, may provide significant economical benefits.

Poster Board Number: 3542

RESPONSES OF HUMAN EMBRYONIC STEM CELLS TO CAMPTOTHECIN-INDUCED DNA DAMAGE

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Human embryonic stem cells (hESCs) possess the unique characteristic of indefinite self-renewal while retaining an undifferentiated state. The reasons for this may include the ability to preserve genomic integrity to a higher degree than somatic cells. Maintaining genomic integrity is vital for stem cells and their function as a primary progenitor, since mutations will severely compromise all derived cell lineages. The pathways controlled by the ataxia telangiectasia-mutated (ATM) and ATM-related (ATR) proteins represent the principal pathways by which cells react to DNA damage in somatic cells. The mechanisms that protect the genome in proliferating hESCs are minimally understood. Here, we investigated how WA09 hESC line react to replication mediated double-strand DNA breaks induced by camptothecin (CPT). Components of the ATM/p53/p21 pathway were studied. Immunofluorescence microscopy revealed the presence of phosphorylated ATM and gamma-H2AX in CPT treated hESCs. A significant decrease in gamma-H2AX foci was observed by treatment with PI3KK inhibitors (caffeine and KU55933). A marked induction of p21WAF1 mRNA levels was determined by qRT-PCR, while p27Kip1 and p57Kip2 transcripts abundance remained unaffected. Importantly, the mRNA levels of Skp2, a member of the SKP1/CUL1/F-box protein complex involved in ubiquitination and proteasomal degradation of p21WAF1, p27Kip1 and p57Kip2 , were reduced upon CPT induced DNA damage. Collectively, our findings demonstrate that the ATM checkpoint signaling cascade is intact in pluripotent hESCs and provide insights into pathways that are activated in human embryonic stem cells upon DNA insult to support development and tissue regeneration.

Poster Board Number: 3544

NUCLEOTIDE-LEVEL RESOLUTION OF LIN28 RNA BINDING SITES REVEALS A NETWORK OF POST-TRANSCRIPTIONAL CONTROL IN HUMAN EMBRYONIC STEM CELLS

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Post-transcriptional control of gene expression is increasingly recognized as critical in the maintenance of pluripotency and differentiation of human embryonic stem cells (hESCs). The RNA binding protein LIN28 is important for the survival and growth of pluripotent cells and increases the efficiency of reprogramming of somatic cells into induced pluripotent stem cells. Additionally, LIN28 has been shown to be overrepresented in primary tumors and cancer cell lines. However, the mechanism by which LIN28 exerts these effects remains unknown. Recent studies using standard immunoprecipitation techniques have shown LIN28 to associate with hundreds of RNA transcripts, and yet the impact of this binding on translation has been validated for fewer than a dozen genes. We hypothesize that revealing the direct RNA targets of LIN28 at nucleotide-level resolution, coupled with high-throughput functional assays to reveal the consequences of this binding, will lead to a greater understanding of the molecular function of this protein. Using UV cross-linking and immunoprecipitation of the LIN28 protein followed by high-throughput sequencing (CLIP-seq) we have identified direct LIN28 binding regions on thousands of target RNAs in hESCs. Strikingly, despite differences in cell types, we can recapitulate our CLIP-seq results using an antibody against the V5 tag of a LIN28-v5 fusion protein stably expressed in human 293FRT cells. We demonstrate that LIN28 preferentially binds coding exons and 3′ untranslated regions (3′ UTRs) within target genes. Gene ontology analyses have revealed that LIN28 targets are enriched for components of the translational machinery and RNA binding proteins, in particular, factors that control alternative splicing. To elucidate the downstream impact of LIN28 modulation on alternative splicing, we are using custom-designed splicing-sensitive microarrays. To confirm the impact of LIN28 on identified RNA binding targets, we are applying RNA-seq, ribosomal profiling and proteomics techniques. This work represents the first combination of these approaches for the study of an RNA binding protein. We expect that the results of these experiments will reveal the global effect of LIN28 binding on transcript stability and translational state of target mRNAs. Our findings will provide insight for the improvement of reprogramming and the role of LIN28 in cancer.

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Poster Board Number: 3546

NPR-C IS REQUIRED FOR SURVIVAL OF MURINE EMBRYONIC STEM CELLS

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Growth of embryonic stem (ES) cells as a pluripotent population requires a balance between survival, proliferation and self-renewal signals. Recently, we found that natriuretic peptides are expressed in ES cells and they play important roles in ES cell self-renewal. Here we report a novel role for natriuretic peptide receptor-C (NPR-C) in the survival of murine ES cells. We found that NPR-C was expressed in undifferentiated ES cells and down-regulated precisely during ES cell differentiation at both mRNA and protein levels. Furthermore, NPR-C was co-expressed with Oct-4 in the blastocyst inner cell mass (ICM). A small interfering RNA (siRNA)-based technique was employed to specifically knockdown NPR-C gene in the undifferentiated ES cells that were maintained in a feeder-free culture. Abrogation of NPR-C signaling had no effect on the undifferentiated status of the ES cells, as determined by morphologic examination and confirmed by measurements of alkaline phosphatase activity and the expression of the pluripotent ES cell markers (Oct-4 and nanog). However, knockdown of NPR-C resulted in apoptotic cell death, and induction of p53. Conversely, chemical inhibition of p53 significantly reduced apoptosis in NPR-C-deficient cells. Treatment of murine ES cells with a selective NPR-C ligand, prior to H2O2 exposure conferred protective effect against oxidative stress-induced apoptosis in dose dependent manner. Activation of p53 by DNA damaging agents results in suppression of Nanog expression, which was blocked by the pretreatment with NPR-C agonist. These findings establish the presence of functional NPR-C in ES cells maintaining their survival through regulation of p53 levels. Thus, NPR-C is required to control DNA damage-induced p53 levels for sustainable ES cell self-renewal.

Poster Board Number: 3548

MODELLING MOUSE EMBRYONIC STEM CELL SELF-RENEWAL AT DIFFERENT O2 LEVELS USING A FACTORIAL DESIGN APPROACH

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Mouse embryonic stem (mES) cells can self-renewal in feeder-free culture conditions via activation of STAT3 signalling by the leukemia inhibitor factor (LIF) and via Smad pathway activation by the bone morphogenetic protein (BMP-4). Alternatively, undifferentiated mES cells can be expanded independently of the activation of the STAT3/Smad signalling via the inhibition of the mitogen-activated protein kinase (MAPK, MEK/ERK signaling pathway) and inhibition of the glycogen synthase kinase 3β (GSK-3β) using the small molecules PD0325901 and CHIR99021, respectively.
Work previously performed in our group showed that culturing mES cells under different oxygen tensions gave origin to different cell proliferation patterns and commitment stages dependent on which signalling pathways are activated/inhibited to support mES cells self-renewal. These results show that mES cell self-renewal and pluripotency, can be differently influenced by the O2 tension levels according to the effect exerted by this culture parameter towards the different signalling pathways. To further investigate these effects more systematically, the sole and interactive influence of each one of the small molecules used for the inhibition of MEK/ERK pathway (PD0325901, at 0, 0.4 and 0.8 μM), activation of Wnt/β-Catenin pathway through the inhibition of GSK-3β (CHIR99021, at 0, 3 and 6 μM) and activation of the STAT3 signalling (LIF, at 0, 500 and 1000 U/mL), was statistically evaluated for the *ex vivo* expansion of 46C mES cells. Both the cumulative fold increase (CUM_FI) after 5 consecutive passages (10 days) and the colony forming efficiency were used as the read-out for this systematic study. A two-level face-centered cube design experiment was performed at 2 and 20% oxygen levels using as basal medium the knockout Dulbecco's modified eagle's medium (DMEM; Gibco) supplemented with 15% (v/v) knockout serum-replacement. Our results reinforce that LIF/STAT3 signaling pathway has a central role in maintaining mES cell proliferation at normoxic conditions. Hypoxia reduces significantly the cumulative fold increase of these cells, which accomplish its maximum CUM_Fi at 2% O2 when the Wnt/β-Catenin pathway is activated in the presence of LIF. In addition, we showed that we could propagate mES cells with complete bypass of the MEK signaling pathway. Our study also suggests that mES cells pluripotency is maximized, as evaluated by the clonal efficiency assay, only when either the MEK signaling or the Wnt/β-Catenin pathway are activated in normoxia in the presence of LIF. This suggests that the MEK /ERK signaling inhibition might interact upon the activation of Wnt/β-Catenin pathway promoting the improvement of mouse ES cell pluripotency in normoxia. At lower O2 tensions, however, STAT3 signaling and Wnt/β-Catenin are the two main and indispensable signaling pathways required for mES cell self-renewal and pluripotency. Collectively, the present data adds new insights into the mechanisms by which the oxygen tension influences mES cell self-renewal and pluripotency while distinct pathways are activated/inhibited. Wnt/β-Catenin, in particular, has an important reported role towards maintenance of both mouse and human ES cell pluripotency, which increases the possibility of translating these findings to human pluripotent stem cells envisaging the maximization of cell yield.

Poster Board Number: 3550

FOLLISTATIN ISOFORMS REGULATE MOUSE EMBRYONIC STEM CELL SELF-RENEWAL

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Introduction: Follistatin (fst) is a protein that is able to bind and antagonize members of TGF-β superfamily, including activins and bone morphogenetic proteins (BMP). As activins and BMPs play an important role in embryonic stem (ES) cell self-renewal and pluripotency, it is likely that follistatin is also involved in these important characteristics of ES cells. Follistatin isoforms arise from alternative pre-mRNA splicing and proteolytic cleavage and exist as three isoforms (i.e., fst288, fst303 and fst315) differing in length at the C terminus. In this work, we try to delineate the roles of follistatin isoforms in mouse ES cell self-renewal and pluripotency. Methods: We constructed four fst isoform-specific transgenes (fst288, fst303, fst315 and fst-myc), which are drived by the EF1 promoter and can consistently overexpress follistatin isoform 288, 303 and 315, respectively. The fst-myc was fst315 with a myc-tag linked at its C terminus, which design may be able to prevent fst315 from proteolysis into fst303 *in vivo*. Mouse ES cells were transfected with these four transgenes individually by electroporation, and then stable clones were screened. To test the influence of follistatin isoforms on self-renewal, mouse ES cell clones were cultivated in various conditions with different growth factors. During cultivation, the percentage of Oct4 positive ES cells was determined by flow cytometry. In the meanwhile, expression levels of self-renewal marker genes (Oct4, Nanog, Rex1, Sox2 and alkaline phosphatase) were evaluated by qRT-PCR. Passage survival of different ES cell clones under various conditions was also observed. Assessments of cell proliferation and apoptosis were performed by flow cytometry for cell counting, BrdU incorporation, Annexin V-7 ADD staining and TUNEL. To determine the effect of follistatin isoforms on pluripotency, embryoid body (EB) differentiation (day 0, 3, 9, 15 21 and 27) was made. The samples were

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then used for analyses of early and late endodermal, mesodermal and ectodermal markers by qRT-PCR and immunostaining. Studies into the potential interaction of follistatin isoforms with specific signalings (LIF, BMP, Act, PI3K/Akt and Wnt) were conducted by Western blots. Results: In the presence of LIF but without feeders, fst288, fst303, fst315 and fst315myc can be propagated for 4 passages, compared with mES that can be propagated for at least 6 passages. Follistatin isoforms assume different effects on proliferation. Follistatin isoforms appear to reduce apoptosis of mouse ES cells in absence of LIF and serum. Follistatin isoforms did not block the endodermal, mesodermal, or ectodermal commitments, however, with different extents of diverse gene expressions during EB differentiation. Conclusions: Follistatin isoforms regulate mouse ES cell self-renewal and pluripotency with distinct responses to different culture conditions.

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A GENOME-SCALE SIRNA SCREEN IDENTIFIES NOVEL REGULATORS OF PLURIPOTENCY AND DIFFERENTIATION IN NANOG-REPORTER MOUSE EMBRYONIC STEM CELLS UNDER DIFFERENTIATION CONDITIONS

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In order to better understand the molecular basis of stem cell pluripotency while using an unbiased and comprehensive approach, we used a genome-scale siRNA library to screen for genetic pathways critical for maintaining pluripotency and differentiation in mouse embryonic stem (ES) cells. Because of its critical role in regulating and maintaining pluripotency and the lack of genome-scale studies on modulators of the particular gatekeeper Nanog, we utilized a Nanog-promoter GFP-reporter transgenic mouse ES cell line. In order to permit a two-way readout to screen for pathways that either enhance or block differentiation, we carried out our siRNA screen in the presence of mild differentiation conditions containing low concentrations of the morphogen retinoic acid, permitting primarily neuronal cell fates. We utilize a novel cell-level analysis to explain the effect of an siRNA pool targeting one specific gene of the distribution of fluorescence. Our principal-component-based analyses directly identify novel genes which affect ES cell pluripotency characteristics in the Nanog reporter system as well as genes which might affect the distribution of fluorescence without affecting the average level in a population of cells. Our screen results correlate well with published work and identify numerous previously characterized pluripotency mediators as well as novel regulators. We are in the process of validating these hits, first by assaying the candidate hit genes in a demultiplexed arrayed plate with one siRNA targeting each gene in our existing reporter, followed by immunostaining for additional pluripotency regulators and lineage markers. In parallel, we are constructing inducible shRNAmir lentiviruses to downregulate candidate genes as well as inducible overexpression systems, focusing on related genes with strong and opposite effects on the reporter in the primary screen or genes with differential expression or promoter occupancy in the differentiated versus undifferentiated state. Collectively, such systems will allow us to characterize the genetic and epigenetic profile of an ES cell in response to multiple mediators of differentiation and inform our understanding of embryonic development and the regulation of pluripotency.

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INVESTIGATION OF STEM CELL SPECIFIC MIRNA EXPRESSION IN MOUSE AND RABBIT EMBRYONIC STEM CELLS

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miRNAs are emerging regulators of gene expression, predicted to target about one third of all human genes. Structurally, miRNAs are short, 22-26 nt single stranded RNA molecules. Though they have been described to regulate gene expression at multiple levels, in mammalian cells they mainly act by blocking the translation of mRNAs (targets) with partially complementer recognition sequences in their 3′ UTR. Embryonic stem (ES) cell-specific miRNAs are abundant in the pluripotent stem cells, but rapidly disappear during differentiation. Using conditional knock-out ES cell lines, disrupted in miRNA biogenesis (Dicer and DGCR8 knock-out ES cell lines), it is now evident that miRNA pathway is a compulsory regulator of pluripotency. miRNAs are inevitable for the maintenance of high proliferation and the cell cycle phase distribution, characteristic to pluripotent ES cells. Moreover, it is now clear that ES-specific miRNA promoters are regulated by common stem cell markers, such as Oct4, Sox2 and Nanog. On the other hand, ESC-specific miRNAs can directly target these transcription factors, establishing a complete negative feedback loop, which is a key regulator of pluripotency maintenance. The best studied ES cell-specific miRNAs are: miR302-367, miR-371-373 and miR-512-519 family in human ESCs; and miR-290-295 family in mice. Initially, our experiments focused on the functional characterization of miR-290-295 cluster. We provide evidence that mouse ES cell lines, stably overexpressing miR-290-295 cluster, have increased rate of cell proliferation and colony formation. We have also shown that miR-290-295 overexpression interferes with early differentiation events. Our results suggest that the cluster regulates cell cycle at multiple steps (G1-S transition and G2-M transition), by directly inhibiting the translation of at least two cell cycle regulators: Fbxl5 and Wee1. In summary, we suggest that miR-290-295 cluster is an important regulator of pluripotency maintenance and exerts its effect by directly inhibiting several cell cycle suppressors. Lately we extended our research interest towards rabbit derived ESCs and early embryos. Rabbit ESCs present high expression of a miR-302a, miR-302b and miR-367 while miR-294 expression is somewhat lower. We could not detect mature miR-290, miR-295, miR-371 or miR-373 miRNAs, using the primers optimized for mouse of human sequences, probably because of the inadequate sequence similarities. The SOLiD Sequencing System allowed us to identify numerous miRNAs that are specific for rabbit ES cells. The newly described miRNAs have already been validated on rabbit embryos and ES cells. We anticipate that knowing the miRNA signature of rabbit ES cells will explain and solve the problem of ES cell isolation from different species. To this end, we will also explore the stemness related targets of miR-302-367 and miR-290-295 families; including in silico analysis and target validation with luciferase assay and at protein level via Western blot analysis or ELISA.

Poster Board Number: 3556

WNT/β-CATENIN SIGNALING NEGATIVELY REGULATES ERK PATHWAY IN MOUSE EMBRYONIC STEM CELLS

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Several signaling cascades as well as the expression of intrinsic factors, such as Oct3/4 and Nanog, maintain self-renewal and pluripotency of embryonic stem cell (ESC). The signaling cascades are activated by extrinsic factors, such as LIF, BMP and Wnt. Heparan sulfate (HS) is a ubiquitous component of proteoglycans in the extracellular matrix and on the cell surface. HS

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polysaccharide chains are attached covalently to Ser residues in core proteins through the linkage tetrasaccharides, GlcAβ1-3Galβ1-3Galβ1-4Xyl. HS are synthesized in the Golgi apparatus by several enzymes, including members of the EXT protein family, which elongate HS chain by adding repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, (-4GlcAb1-4GlcNAcα1-)n. These repeating units are then modified by sulfates and are known to play crucial roles in regulating several signaling cascades. Previously, we have demonstrated that Wnt signaling was reduced by knockdown of EXT1 in mouse ESCs (mESCs) and that, subsequently, spontaneous differentiation into extraembryonic endoderm was induced. FGF4 signaling has been reported to trigger the induction to the differentiation in mESCs. To clarify the mechanism of spontaneous differentiation in EXT1 knockdown cells, we analyzed the responsivity of EXT1 knockdown cells to exogenous FGF4 and found that their responses to FGF4 stimulation did not change as compared with that of control cells. Surprisingly, internal activation of ERK was higher in EXT1 knockdown cells than control cells. Therefore, we hypothesized that the activation of ERK in EXT1 knockdown cells induces spontaneous differentiation. To test this hypothesis, we added the MEK inhibitor to EXT1 knockdown cells and made their ERK phosphorylation decrease. Then self-renewal of EXT1 knockdown cells was restored. These results indicated that spontaneous differentiation was caused by activation of ERK pathway. The only Wnt/β-catenin signaling was down-regulated in EXT1 knockdown cells. Hence, ERK pathway was activated and spontaneous differentiation was induced because of the reduction of Wnt/β-catenin signaling. We propose that Wnt/β-catenin signaling negatively regulates ERK pathway in mESCs and HS chains are involved in this regulation.

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SHEAR STRESS IN STIRRED SUSPENSION BIOREACTORS INFLUENCES PLURIPOTENCY OF MURINE EMBRYONIC STEM CELLS

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Pluripotent embryonic stem cells (ESCs) have been used increasingly in research as primary material for various tissue engineering applications. Pluripotency, or the ability to give rise to any cells of the body, is an important characteristic of ESCs. Traditional methods use leukemia inhibitory factor (LIF) to maintain murine ESC (mESC) pluripotency in static and bioreactor cultures. When LIF is removed from mESCs in static culture, pluripotency genes are down-regulated and the cultures will spontaneously differentiate. However, recently, we showed that shear forces experienced by mESCs in suspension bioreactors may influence pluripotency. Experiments conducted within suspension bioreactors indicated that pluripotency gene expression was maintained during differentiation experiments in the absence of LIF. This is undesired in a differentiation experiment where the goal is down-regulation of pluripotency gene expression and up-regulation of gene expression characteristic to the differentiation. Thus, the objective of this study was to examine how effectively different levels of shear (6 dyne/cm2, 3 dyne/cm2) maintained and rescued mESC pluripotency in suspension bioreactors. Pluripotency markers, Oct-4, Nanog, Sox-2, and Rex-1, were assessed using gene expression profiles and flow cytometry analysis and showed that shear does maintain and rescue the gene expression of certain pluripotency markers. For example, in the shear maintenance experiment derived from static cultures, the gene expression of Rex-1, and Sox-2 were effectively maintained by both levels of shear stress. Overall, this study provides a better understanding of the environmental conditions within suspension bioreactors, and specifically how different levels of shear affect the pluripotency of ESCs.

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COOPERATION OF PKA/RHOA AND EPAC/RAP1 SIGNALING IN 8-BROMO CAMP-INDUCED CX43 PHOSPHORYLATION IN MOUSE EMBRYONIC STEM CELLS

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Gap junctions play multiple essential roles in cell homeostasis and connexin (Cx) phosphorylation affects both the structure and function of Cx43 and leads to changes in localization, interacting protein partners and channel selectivity in response to many different kinases. However, the impact signaling in response to cAMP stimuli is less well understood in embryonic stem (ES) cells. Thus, we examined effect of 8-Bromo (8-Br) cAMP on Cx43 phosphorylation of ES cells and its related signaling pathways. 8-Br cAMP increased Cx43 phosphorylation and decreased Cx43 protein level in plasma membrane compartment. Moreover, 8-Br cAMP inhibited transfer of Lucifer yellow to neighboring cells in a scrape loading/dye transfer (SL/DT) assay. Thus, we further examined the molecular mechanisms responsible for the phosphorylation of Cx43 by 8-Br cAMP. 8-Br cAMP stimulated PKA/Rho A and Epac/Rap1 signaling pathways, respectively. Moreover, inhibition of either Rho A or Rap1 signaling pathway partially blocked 8-Br cAMP-induced Cx43 phosphorylation and decrease of Cx43 protein level in membrane compartment, but both inhibition completely blocked. Furthermore, 8-Br cAMP-induced Cx43 phosphorylation and Cx43 protein endocytosis disrupted interaction of Cx43 with tight junctional proteins such as occludin, claudin, and Zo-1 or adherent junctional proteins such as catenin and E-cadherin in plasma membrane compartment, which were mediated by Rho A and Rap1 signaling pathways. This suggests that Cx43 structural changes are contributed to cell adhesion formation. In conclusion, 8-Br cAMP stimulated Cx43 phosphorylation via PKA/Rho A and Epac/Rap1 signaling pathways, which regulated ES cell adhesion.

Poster Board Number: 3562

MAINTENANCE OF GENOMIC IMPRINTING IN MOUSE EMBRYONIC STEM CELLS

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Zfp57 is highly expressed in undifferentiated ES cells and down-regulated during ES cell differentiation. It encodes a KRAB zinc finger protein and is conserved between mouse and human. Previously, we found that Zfp57 is a maternal-zygotic effect gene and is required for the maintenance of DNA methylation imprints in mouse embryos. Interestingly, ZFP57 was shown to be associated with an imprinted domain in ES cells in a chromatin immunoprecipitation (ChIP) assay. Indeed, our recent data indicate that loss of Zfp57 in ES cells results in loss of DNA methylation genomic imprints at a large subset of imprinted regions, including the Dlk1-Dio3 imprinted domain. Zfp57 is also required for the maintenance of genomic imprinting in mouse ES cells directly derived from blastocysts. Our results have confirmed that Zfp57 is an important regulator of genomic imprinting in ES cells. Intriguingly, improper expression of the imprinted genes at the Dlk1-Dio3 imprinted domain is the notable epigenetic defect present in a majority of mouse iPS cell lines. We are developing several different strategies to examine if genomic imprinting can be manipulated in ES cells and iPS cells via Zfp57-mediated regulation.

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A PANORAMIC VIEW OF NON-POLYADENYLATED TRANSCRIPTION IN MOUSE ESC AND NPC REVEALS NOVEL NON-CODING RNA RELATED TO PLURIPOTENCY AND DIFFERENTIATION

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Hypertranscription in ESCs is a characteristic underlying the pluripotent nature of these cells. Yet, this characterization results mainly from the examination of the polyadenylated fraction of cellular RNA, while the non-polyadenylated (NPA) fraction remains largely unexplored. However, the NPA RNA superfamily, which is mainly comprised of fundamental components of the RNA translational machinery, the RNA splicing machinery, and RNA-RNA or RNA-DNA interacting complexes, has every potential to participate in the regulation of pluripotency and stem cell fate. We conducted a comprehensive analysis of the transcriptive nature of NPA RNA in murine ESCs and ESC-derived Neural Progenitor Cells (NPCs) using a combination of whole genome tiling arrays and Next Generation Sequencing technologies. This strategy allowed for the transcriptional characterization of already well-defined NPA RNA subclasses in this unique biological context as well as the identification of many new members of these functional ncRNA classes. In addition, many novel, conserved, and differentially expressed NPA RNAs were detected, providing the first evidence of a novel group of potentially functional ncRNAs involved in the regulation of pluripotency and stem cell fate.

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LACDINAC CARBOHYDRATE STRUCTURE CONTRIBUTES TO SELF RENEWAL OF MOUSE EMBRYONIC STEM CELLS BY REGULATING LIF/STAT3 SIGNALING

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Self-renewal of mouse embryonic stem cells (mESCs) is maintained by leukemia inhibitory factor (LIF)/STAT3 signaling. However, this signaling control does not function in mouse epiblast stem cells (mEpiSCs) or human ESCs (hESCs), including human induced pluripotent stem cells (hiPSCs). The underlying molecular mechanisms that determine this differential LIF-responsiveness have not been clarified yet. Several types of glycan are expressed on the cell surface and contribute to biologically important functions such as the regulation of signaling pathways. The expression patterns of several cell surface glycans have now been described in undifferentiated and differentiated mESCs and hESCs. However, their functional roles have not been demonstrated with the exception of heparan sulfate. Therefore, to identify glycans that were essential for ESC self-renewal, we performed an RNA interference (RNAi) screen using short hairpin RNAs (shRNAs) that targeted specific glycan-related genes in mESCs by evaluation of alkaline phosphatase activity. Then we found that the cell surface glycan LacdiNAc (GalNAcβ1-4GlcNAc) is required for LIF/STAT3 signaling. LacdiNAc is frequently present on glycoproteins and glycolipids in invertebrates but is only present on a limited number of glycoproteins and glycolipids, such as glycoprotein hormones, in vertebrates. The roles of LacdiNAc in mammalian cells have not been fully understood. Undifferentiated state mESCs expressed LacdiNAc at a higher level than differentiated state cells. Knockdown of β4GalNAc-T3 reduced LacdiNAc expression and caused a decrease in LIF/STAT3 signaling that lessened the rate of self-renewal of mESCs. A biochemical analysis showed that LacdiNAc expression on LIFR and gp130 was required for the stable localization of the receptors with lipid raft/caveolar components, such as caveolin-1. This localization is required for transduction of a sufficiently strong LIF/STAT3 signal. In primed state pluripotent stem cells, such as hiPSCs and mEpiSC-like cells produced from mESCs, LacdiNAc expression on LIFR and gp130 was extremely weak and the level of localization of these receptors on rafts/caveolae was also low. Furthermore, knockdown of β4GalNAc-T3 decreased LacdiNAc expression and reduced the efficiency of reversion of primed state mEpiSC-like cells into naïve state mESCs. These findings demonstrate that the different LIF-responsiveness of naïve state (mESCs) and primed state (mEpiSCs, hESCs, hiPSCs) cells is dependent upon the expression of LacdiNAc on LIFR and gp130 and that this expression is required for the induction and maintenance of the naïve state.

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RAD21 COOPERATES WITH PLURIPOTENCY TRANSCRIPTION FACTORS IN THE MAINTENANCE OF EMBRYONIC STEM CELL IDENTITY.

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For self-renewal, embryonic stem cells (ESCs) require the expression of specific transcription factors accompanied by a particular chromosome organization to maintain a balance between pluripotency and the capacity for rapid differentiation. However, how transcriptional regulation is linked to chromosome organization in ESCs is not well understood. We show that the cohesin component RAD21 exhibits a functional role in maintaining ESC identity through association with the pluripotency transcriptional network. ChIP-seq analyses of RAD21 reveal an ESC specific cohesin binding pattern that is characterized by CTCF independent co-localization of cohesin with pluripotency related transcription factors Oct4, Nanog, Sox2, Esrrb and Klf4. Upon ESC differentiation, most of these binding sites disappear and instead new CTCF independent RAD21 binding sites emerge, which are enriched for binding sites of transcription factors implicated in early differentiation. Furthermore, knock-down of RAD21 causes expression changes that are similar to expression changes after Nanog depletion, demonstrating the functional relevance of the RAD21 - pluripotency transcriptional network association. Finally, we show that Nanog physically interacts with the cohesin or cohesin interacting proteins STAG1 and WAPL further substantiating this association. Based on these findings we propose that a dynamic placement of cohesin by pluripotency transcription factors contributes to a chromosome organization supporting the ESC expression program.

Poster Board Number: 3570

REVERSION OF MOUSE EPIBLAST STEM CELLS TO NAIVE PLURIPOTENT CELLS BY EXOGENOUS REACTIVATION OF DOWN-REGULATED SIGNALING PATHWAYS

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Mouse embryonic stem cells (mESCs) and mouse epiblast stem cells (EpiSCs) are pluripotent stem cells that are derived from developmentally related tissues in the early mouse blastocyst. *In vitro*, mESCs and EpiSCs are capable of transitioning bidirectionally. Going forward, mESCs can readily differenti-

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ate into culture derived EpiSCs (CDEs) upon mimicking *in vivo* conditions (i.e. 3D structure + FGF signaling) and subsequent culture in EpiSC-specific media (Activin A + bFGF or AF media). Reprogramming EpiSCs to mESCs-like cells, however, requires overexpression of transgenes. Alternatively, transgene-free reprogramming of EpiSCs can occur on a layer of feeder cells where JAK-STAT signaling plays an important role. However the mechanisms facilitating this rare reprogramming event remain obscure. We have previously demonstrated that the JAK-STAT pathway in mESCs functions as a feed-forward autoregulatory loop. Interestingly, upon differentiation to CDEs, mESCs lose responsiveness to leukemia inhibitory factor (LIF) due primarily to downregulation of its receptors, LIF-R and gp130 (10-fold and 3-fold respective decrease by qRT-PCR), and subsequent degradation of activated (i.e. phosphorylated) STAT3 (pSTAT3). We hypothesized that re-activation of STAT3 by bypassing its dependence on LIF-R would enable reprogramming of EpiSCs to naïve mESC-like cells. Indeed, bypassing LIF-R by using IL-6 along with its soluble receptor, sIL-6Rα, increased pSTAT3 levels in regions of high local cell density. We were also able to obtain, from this pre-treatment, reverted colonies at low frequency (1 for every 75,000 cells seeded) when placed in defined feeder-free naïve mESC media (2i + LIF media). We further hypothesized that an increase in local cell density leads to a concomitant increase in both the proportion of LIF responsive cells and the frequency of reversion. Consistent with this, we observed increased local cell density, LIF responsiveness, and reversion frequencies in CDEs cultured on feeders (MEFs). We next controlled local cell density by micropatterning (μP) CDEs in the absence of feeders and observed an increase in LIF responsiveness and importantly, in frequency of reversion (3 colonies for every 10,000 cells seeded), demonstrating the ability to recapitulate conditions seen on MEFs. The reverted cells displayed gene expression profiles and protein levels comparable to mESCs but not CDEs, and were able to generate chimeras when injected into host blastocysts. The increase in local cell density leads to an increase in LIF responsiveness in part due to accumulation of local gp130 ligands, as inhibition of JAK-STAT signaling on μP colonies abolishes LIF responsiveness. Our findings suggest controlling local cell density, and its resultant autocrine and paracrine signaling, facilitates reprogramming of EpiSCs through reactivation of suppressed JAK-STAT signaling, demonstrating for the first time efficient feeder and transgene-free reversion. More generally, these results demonstrate that transitions between closely related cell types may be accessible via exogenous reactivation of differentiation-induced downregulation of signaling pathways.

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FIBRONECTIN STIMULATES CELL MIGRATION THROUGH NHE-1 ACTIVATION VIA CAVIN-1 AND CALMODULIN COMPLEX IN MOUSE EMBRYONIC STEM CELLS

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Recent works have focused on the possible role of Na+/H+ exchanger isoform 1 (NHE-1) as a signaling complex, due to its function of anchoring for cytoskeleton and scaffolding for signaling protein; however a lot remains to be evaluated regarding role of NHE-1 in the control of embryonic stem (ES) cell migration. Thus, this study has examined the effect of fibronectin (FN) on NHE-1 and its relationship to migration of mouse ES cells. FN including other ECM components (laminin, fibrinogen, or collagen type I) increased Na+ uptake, but their effects were less than FN. Co-immunoprecipitation (Co-IP) studies demonstrated that FN induced formation of complexes between cavin-1 and calmodulin (CaM), as well as between CaM and NHE-1. These results revealed that there was a marked increase in the amount of cavin-1 and NHE-1 in CaM immunoprecipitates after FN treatment in a time- dependent manner, which were blocked by integrin β1 neutralizing antibody, integrin β1 siRNA, methyl-β-cyclodextrin (MβCD, lipid raft disruptor), or cavin-1 siRNA. In addition, pretreatment of these inhibitors blocked the FN-induced increase of Na+ uptake. FN increased Ca2+ influx and PKC activation and Co-IP levels of PKC and CaM, which were also contributed to FN-induced NHE-1 activation. Finally, FN significantly increases RhoA expression in a time (≥ 12 h)- dependent manner, which was blocked by amolide (NHE inhibitor) and transfection of NHE-1 siRNA. We further identified that FN stimulated ES cell migration via increase of MMP-2 and F-actin expression levels. These effects were inhibited by GGTI-286 (RhoA inhibitor) or Y-27632 (ROCK inhibitor). In conclusion, FN stimulated ES cell migration through NHE-1 activation via cavin-1/camodulin complex formation.

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ENHANCING HOMOLOGOUS RECOMBINATION BY ALTERING CULTURE CONDITIONS IN MOUSE ES CELLS

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Embryonic stem cells are characterized by their unique properties of self-renewal and pluripotency. Also these cells exhibit relatively higher frequencies of homologous recombination in comparison to the primary somatic cells. One of the promising therapeutic potential of ES cells to treatment of some of the genetic disorders by correcting the mutation in ES cells and then using these ES cells for stem cell therapy. Though ES cells show relative higher frequencies of homologous recombination. The process is still inefficient and laborious. We have used various culture conditions to study the efficiency of homologous recombination in Hprt locus in mouse ES cells. The conditions included commercially available media, conditioned media and small molecules. We have few identified conditions in which, the frequency of homologous recombination was enhanced by 5-10 times relative to the ES cells cultured in regular ES cells medium containing 15% ES cell tested FBS. Our results show that the culture conditions can influence the frequency of homologous recombination and the frequency can be improved under appropriate culture conditions. We are performing experiment with targeting vectors targeting other loci in mouse genome to ensure that this phenomenon is not specific to Hprt locus.

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EXTRACELLULAR MATRIX-BASED SIGNALING PROMPTS AN EXIT FROM THE NAÏVE PLURIPOTENT MOUSE STEM CELL STATE IN THE ABSENCE OF CELL-SECRETED SIGNALS

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Cell-secreted signals are an integral part of the extracellular environment of embryonic stem cells, but their precise contributions to cell phenotype are often difficult to define. While it is known that cell-secreted factors play a role in embryonic stem cell (ESC) growth, self-renewal, and differentiation, the role of such signals in these processes is only starting to be uncovered. Here, we present a microfluidic perfusion method that continuously removes soluble cell-secreted proteins. We use this method to show that soluble signaling is required for self-renewal of mouse ESCs, and that without this signaling the cells exit their self-renewing state and enter a more epiblast-like, primed pluripotent state. Upon removal of soluble signals under perfusion, ESCs enter a defined lineage in which cells downregulate the ESC markers Rex1 and Klf4 and upregulate the EpiSC markers Fgf5, Brachyury, and Dnmt3b. These epiblast-like cells are dependent on Activin for Nanog expression, as they are refractory to LIF signaling, and they can be propa-

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gated in defined epiblast stem cell media. This state change is surprising given the fact that LIF and BMP4 are still present in the culture media of the cells grown under perfusion, factors which together have been thought to be sufficient for maintenance of ESC self-renewal. Thus, our results show that LIF and BMP4 are not sufficient to maintain the self-renewal of mESCs in the absence of soluble signaling and thus that cell-secreted factors play a critical role in self-renewal. Removal of soluble cell-secreted factors by perfusion also uncovers the function of non-diffusive factors, including many components of the extracellular matrix. We show that the continued presence of functional ECM is responsible for the exit from the ESC state, as disruption of the ECM by attenuation of heparan sulfate proteoglycan function mitigates the state change. Intriguingly, the major contribution of the ECM that drives cells toward an epiblast-like fate is not Fgf4 signaling through ERK, as inhibition of this signaling pathway under perfusion does not halt the phenotypic change. Since our results suggest that ECM remodeling is critical for mESC self-renewal, we investigated the role of matrix metalloproteinases (MMPs) in this process. MMPs are the major class of cell-secreted proteins responsible for curating the ECM, and thus are appealing candidates for the cell-secreted molecules being removed under perfusion. We first assessed whether MMP downregulation by inhibition in static culture would also cause a differentiation phenotype, and indeed we show that when MMPs are inhibited, mESCs are unable to self-renew for more than two passages. Conversely, MMP addition helps to maintain ESC self-renewal, further showing the importance of large-scale ECM remodeling in the self-renewal process. Thus, we are able to uncover a previously undescribed level of extracellular regulation that is involved in maintenance of mESC self-renewal.

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L-THREONINE REGULATES CYCLINS OF MOUSE EMBRYONIC STEM CELLS THROUGH PI3K/AKT, MAPKS, AND MTORC PATHWAYS

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Amino acids are able to control many physiological functions and are involved in regulating early embryonic development. In addition, it is now widely accepted that amino acids can stimulate signal transduction and function as signal molecules regulating many embryonic stem cell (ESC) functions. Nevertheless, amino acid-dependent regulation of ESC function and related signal pathways has not been described. Thus, we investigated the effect of L-threonine on regulation of mouse (m)ESC self-renewal and related signaling pathways. Depletion of L-threonine decreased the expression of undifferentiation marker genes (Oct4, nanog, FOXD3, Rex1, and Sox2) and proliferation (cyclins D1/CDK4 and cyclin E/CDK2) of mESC, which were restored by L-threonine addition. Disruption of the lipid raft/caveolae microdomain through treatment with methyl-β-cyclodextrin or transfection with caveolin-1 specific small interfering RNA blocked L-threonine-induced proliferation of mESCs Addition of L-threonine induced phosphorylation of Akt, ERK, p38, JNK/SAPK, and mTOR in a time-dependent manner. This activity was blocked by LY 294002, wortmannin, or an Akt inhibitor. L-threonine-induced activation of mTOR, p70S6K, and 4E-BP1 as well as cyclins and Oct4 were blocked by PD 98059, SB 203580 or SP 600125. Furthermore, L-threonine induced phosphorylation of raptor and rictor binding to mTOR was completely inhibited by 24 hr treatment with rapamycin; however, a 10 min treatment with rapamycin only partially inhibited rictor phosphorylation. L-threonine induced translocation of rictor from the membrane to the cytosol/nuclear, which blocked by pretreatment with rapamycin. In addition, rapamycin blocked L-threonine-induced increases in mESC proliferation. In conclusion, L-threonine stimulated ESC G1/S transition as well as maintenance of undifferentiated states through lipid raft/caveolae-dependent PI3K/Akt, mTOR, and p70S6K signaling pathways

Poster Board Number: 3580

NOVEL E3 UBIQUITIN LIGASE RNF12 REGULATES FEMALE X-CHROMOSOME INACTIVATION BY DEGRADATION OF OCT4

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Embryonic stem cells (ESCs) are maintained by defined transcription factors including Oct4, Nanog and other stemnness factors. During the differentiation, X-chromosome inactivation (XCI) was appeared in female ESCs for equalization of X-chromosome-linked gene dosage. XCI is regulated by homologous X-chromosome pairing and counting, and random X-chromosome inactivation. Pluripotency factor Oct4 interacts with Ctcf and then controls XCI by their association. Recent report shows that XCI is determined by Rnf12 expression in a female-dependent manner. Rnf12, an E3 ubiquitin ligase known as Rlim, locates to several killobase near the Xist RNA in X-chromosome and contains ring domain as an E3 activity. Here we demonstrate that Rnf12 functions as E3 ligase for Oct4 and regulates XCI. We found that Rnf12 and Oct4 co-localized to the nuclei in ESCs and Rnf12 targeted Oct4 by ubiquitination in a dose-dependent manner. Rnf12 and Oct4 formed complexes *in vitro* and *in vivo*, especially homeo domain of Oct4 was ubiquitinated by Rnf12 and required for the association with Rnf12. Oct4 was elevated significantly when Rnf12 was disrupted by loss-of-function such as expressions of shRNA and dominant negative mutants. Furthermore, Rnf12 determined XCI by Oct4 degradation. Remarkably, gain-of-functions of Rnf12 activated XCI, but loss-of-functions of Rnf12 decreased XCI. In addition, Rnf12 regulated the stemness and ES differentiation of ESCs by protein levels of Oct4 in wild type or dominant negative Rnf12-expressing ESCs. In conclusion, our findings identify that Rnf12 initiates XCI by degradation of negative XCI regulator Oct4 and activates ES differentiation.

Poster Board Number: 3582

A SHRNA FUNCTIONAL SCREENING OF KINASE AND PHOPHATASE IN ESC RENEWAL

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ESCs provide a prominent model for studying cell renewal, oncogenicity, and pluripotency. Kinases and phosphatases are well-recognized for their importance in cell-fate determination. However, their roles in ESCs remain elusive. To address this issue, we used 4801 shRNAs against 929 kinases and phosphatases to simultaneously identify the pivotal genes for ESC renewal and pluripotency. Alkaline phosphatase activity (ALP) was chosen as the marker used for the primary screening. Based on the result of ALP assay, among the 4801 shRNAs that target 929 genes, 377 shRNAs which recognized 143 genes were identified as the potential targets . Since ESCs undergo morphological change upon differentiation, thirty-one genes which were targeted by 70 shRNAs were selected based on morphological change. Among them, we further demonstrated that two of the candidate genes, Nme6 (non-metastatic cells 6, protein expressed in nucleoside-diphosphate kinase) and Nme7 (non-metastatic cells 7, protein expressed in nucleoside-diphosphate kinase), are essential for ESC renewal and pluripotency. Interestingly, Oct3/4 and Klf4 were reduced in both Nme6- and Nme7- knockdown ESCs. The differentiation markers of ectoderm, endoderm, and mesoderm are also activated upon the depletion of Nme6 or Nme7. This suggests the pivotal roles for Nme6 and Nme7 in ESC renewal.

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Poster Board Number: 3584

DELINEATING THE ROLE OF TCF3 IN EMBRYONIC STEM CELL SELF-RENEWAL AND DIFFERENTIATION

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In addition to its roles in developmental and cancer related processes, emerging evidence suggests that the canonical Wnt/β-catenin/TCF pathway is also involved in regulating stem cell self-renewal and differentiation. The ubiquitous serine/threonine kinase, Glycogen Synthase Kinase-3 (GSK-3) acts as a negative regulator in the Wnt/β-catenin/TCF pathway. Genetic ablation of both GSK3 α and β (referred to as DKO, for double knockout) in mouse embryonic stem cells (ESCs), renders them incapable of efficiently differentiating into the three germ layers. In an attempt to rescue this differentiation blockade of DKO ESCs, we expressed dominant negative forms of the TCFs (TCF1, LEF1, TCF3 and TCF4), the final effector molecules of this pathway. Surprisingly, although the expression of TCF1, LEF1 or TCF4 was tolerated and resulted in attenuated expression of β-catenin/TCF target gene expression, the expression of dominant negative TCF3 (TCF3DN) resulted in premature cell differentiation/death. Thus, TCF3 appears to play a unique role in the regulation of ESC properties. To clarify the underlying mechanism of TCF3 action in ESCs, we are examining the effects of expressing a dominant negative TCF3 (TCF3DN) in DKO cells using an inducible doxycycline regulated system. Gene expression analyses of DKO-TCF3DN ESCs, relative to DKO and DKO-TCF4DN ESCs, suggests that TCF3DN differentially regulates a specific subset of genes. In addition, the differentiation capabilities of DKO-TCF3DN cells were assessed by using embryoid body and teratoma assays. Furthermore, to more fully understand the mechanism by which TCF3 regulates stem cell properties, we have used an unbiased quantitative proteomic approach to identify proteins that interact with TCF3. The information obtained from these studies provides new insights into the mechanisms through which TCF3 regulates stem cell self-renewal and differentiation.

Poster Board Number: 3586

MYC ACTIVITY CAN BE ELIMINATED IN ES CELLS UNDER 2I CONDITION WITHOUT AFFECTING THEIR INDEFINITE SELF-RENEWAL PROPENSITY

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Indefinite self-renewal is one of the most prominent features of ES cells in which a number of transcription factors are known to be involved in the establishment and the maintenance of this important property. c-Myc protein has been shown to be crucially involved in this task as one of major downstream regulators of LIF-Stat3 signaling cascade. However, it is known that expression levels of c-Myc in inner cell mass cells in blastocysts and ES cells cultured under the ground state condition using specific kinase inhibitors (2i condition) are extremely low, implicating that these cells maintain their prominent features through a c-Myc independent mechanism. To address this question directly, we generated null-ES cells for Max gene which encodes a best characterized partner for all of Myc family proteins comprising of c-Myc, N-Myc and L-Myc. Our analyses reveal that Myc activity can be deprived completely without affecting their ES cell-specific prominent features, although loss of Max gene expression leads to loss of pluripotency and cell viability when the cells are under the conventional culture condition. Thus, these results indicate that, unlike Oct3/4, Myc is not an absolute, but is a context dependent regulator of ES cell status and the crucial requirement is rather restricted to ES cells under empirical culture condition using LIF and serum.

Poster Board Number: 3588

FROM EMBRYONIC STEM CELL TO EPIBLAST

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A series of pluripotent cell types characterizes early mammalian development: the zygote, the inner cell mass, and finally epiblast. Epiblast is a pluripotent cell type, able to generate ectoderm, mesoderm, and endoderm, but has lost the ability to generate most extraembryonic tissues. We are interested in understanding the molecular events that restrict the pluripotential state and promote epiblast differentiation, and will use embryonic stem cells (ESCs) as an easily manipulated *in vitro* model. We hypothesize that an FGF5+ epiblast intermediate will be observed during the differentiation of ESCs toward the neural lineage. FGF5 is an established epiblast marker. qRT-PCR and immunocytochemistry analysis of ESCs undergoing neural differentiation suggest a transient FGF5+ epiblast population is present at around 3-4 days. We aim to isolate ESC-derived epiblast and determine its developmental potential. FGF5 cannot be used for this purpose because it is a secreted ligand and very little is known about the regulatory sequences driving expression of FGF5 in epiblast. We are currently screening for genes that display a similar temporal expression pattern to FGF5, with the hope of identifying cell surface markers or genes that can be used to generate a reporter cell line. Preliminary deep sequencing data verifies the expected temporal pattern of FGF5 expression. Once we are able to isolate ESC-derived epiblast, we will begin to investigate the molecular events regulating transition to epiblast. We hypothesize roles for the FGF, Notch, and Hh signaling pathways. A better understanding of this molecular regulation will aid in efficiently generating ESC-derived material for cell-based therapies.

Poster Board Number: 3590

RONIN'S TRANSCRIPTIONAL CONTROL OF DNA REPAIR IN EMBRYONIC STEM CELLS

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Embryonic stem cells (ES cells) provide a potentially powerful tool for regenerative medicine due to two unique properties. Self-renewal allows them to divide continuously in culture without differentiating, while pluripotency enables them to differentiate into all three germ layers. ES cells also possess a low spontaneous mutation rate that is 100 times lower than that of many differentiated cells. However, the mechanism responsible for this low mutation rate remains to be defined. Recently, we have identified a transcription factor named Ronin that is essential to ES cell self-renewal by transcriptionally regulating its targets via direct recruitment of chromatin modifying enzymes through its interaction with Host cell factor 1 (Hcf1). Here we show Ronin controls a transcriptional program specific to ES cell DNA repair. Mapping promoter binding sites for Ronin in ES cells (using ChIP-Seq technology) indicate it to bind genes involved in multiple DNA repair pathways including nucleotide excision repair, post-replication repair, and homologous recombination. Conditional loss of Ronin in ES cells results in increased sensitivity to DNA damage as well as defects in repair of DNA strand breaks. In contrast, constitutive overexpression of Ronin results in enhanced DNA repair and enhanced survival after DNA damage. Our data suggest Ronin to regulate transcription of its bound DNA repair genes. We propose this regulation to be one component of the mechanism underlying the low mutation rate in ES cells. Ultimately, our studies may give insight into the spontaneous mutation rate of adult stem cell populations and the mutation rate within the developing embryo.

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Poster Board Number: 3592

PRINCIPAL COMPONENTS OF EMBRYONIC STEM CELL PLURIPOTENCY

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To decipher gene regulatory networks, we used systematic knockdown of 97 transcription factors (TFs) and 7 other genes with shRNA in mouse embryonic stem (ES) cells, followed by global gene expression profiling with microarrays. Meta-analysis of these data together with the earlier data obtained by the induction of 50 TFs and the existing genome-wide data on TF binding sites identified the sets of target genes regulated by 23 TFs. Target genes were identified based on their over-representation (Parametric Analysis of Gene Set Enrichment, PAGE) in sets of upregulated or downregulated genes after manipulation of the TF. Most of these TFs (Pou5f1, Sox2, Nanog, Esrrb, Sall4, Btbd14b, Zfp281, Nr0b1, Tcfcp2l1, Stat3, Myc, Trp53, Eed, Phc1, Suz12, Klf4, Cdx2, Eomes, and Gata3) tend to positively regulate the expression of their targets, and only 4 TFs (Rest, Jarid2, Tcf3, and Nr5a2) have predominantly negative effects on their targets. Even well-known repressors, such as Polycomb TFs - Eed, Phc1, and Suz12, positively regulate a large number of their targets. Positive regulation of target genes by Pou5f1, Sox2, Nr0b1, Eed, and Suz12 in normal conditions is reverted to negative regulation when these TFs are over-expressed; a phenomenon previously described as "bell-shaped" response. Gene Ontology (GO) analysis of target genes elucidated the key function of each TF. For example, targets regulated by Rest, Esrrb, Klf4, and Trp53 are enriched in synapse, glycolysis, cytoskeleton, and apoptosis functions, respectively. Principal component analysis shows two main directions of ES cells state change after manipulation of TFs: (1) knockdown of Pou5f1 or Sox2 ("Pou5f1 regulatory module"), or induction of Esx1, Cdx2, Gata3, Sox9, or Tcf3 initiates cell differentiation towards trophectoderm, and (2) knockdown of Esrrb, Sall4, Nanog, Gbx2, Grhl2, Mtf2, Aff1, Tcfap4, or Cdc5l ("Esrrb regulatory module"), or induction of Nrip1 initiates cell differentiation towards epiblast lineage. Differentiation towards trophectoderm is associated with a strong downregulation of target genes of core pluripotency factors Pou5f1 and Sox2, whereas differentiation to epiblast is associated with downregulation of targets of Esrrb and upregulation of targets of Trp53 and polycomb TFs. These findings indicate that the Pou5f1 regulatory module prevents trophectoderm differentiation and Esrrb module prevents epiblast differentiation. Esrrb module is downstream of Pou5f1; thus, active Pou5f1 module is a necessary (but not sufficient) condition for Esrrb activity. Pou5f1 module is independent from Esrrb: Pou5f1 remains active after the repression of Esrrb module and is required for ES cell differentiation towards epiblast.

Poster Board Number: 3594

DISSECTING SELF RENEWAL CIRCUITRY USING PHOSPHO-PROTEOMICS AND SMALL MOLECULE MODULATION OF SIGNALING PATHWAYS

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The maintenance of human embryonic stem cell (hESC) pluripotency requires an intricate balance of signaling factors that promote self renewal and inhibit differentiation inducing signals. Although canonical TGF-β, FGF, and Wnt signals synergize to maintain pluripotency at the transcriptional level, the complex crosstalk between these pathways immediately downstream of receptor activation has remained unexplored. Here we demonstrate that TGF-β activated kinase 1 (TAK1) acts as a central node integrating signals between the TGF-β, MAP kinase, and BMP pathways. Inhibition of TAK1 results in the rapid loss of pluripotency and the highly efficient induction of trophoblast differentiation of hESCs. Furthermore this phenotype is completely dependent on the activity of BMP signals. Using a phosphoproteomic strategy, we show that TAK1 prevents differentiation by positively regulating downstream effectors MEK, p38, and JNK to inhibit autocrine BMP signaling. Surprisingly, TAK1 also positively mediates Smad 2/3 activation, and is necessary for receptor induced phosphorylation of Smad 2/3 by TGF-β. Thus TAK1 functions as a central hub in pathway crosstalk to maintain self renewal and inhibit differentiation inducing signals. Our results highlight the multilayered complexity of self renewal signaling circuitry and implicate TAK1 as a central mediator of the pluripotent state.

Poster Board Number: 3596

X-INACTIVATION STATUS IN FEMALE MONKEY EMBRYOS AND EMBRYONIC STEM CELLS

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X chromosome inactivation (XCI) is believed to be essential mechanism compensating expression levels of X-linked genes between females (XX) and males (XY). In preimplantation mouse embryos, paternal X chromosome is selectively inactivated in the extraembryonic lineage while the inner cell mass (ICM) lineage maintains two active chromosomes (XaXa). Embryonic Stem Cells (ESCs) derived from mouse ICMs retain this unique epigenetic feature. Random XCI takes place in the female ICM lineage after implantation or in ESCs upon *in vitro* differentiation. In contrast, XCI status in human ESCs is not uniform, with majority of analyzed female ESCs displaying non-random XCI. It remains unclear whether human ESCs represent developmentally more advanced stages than their mouse counterparts or human ESCs simply inherit XCI status present in the parental ICMs. To date, XCI status of human preimplantation embryos, particularly in the ICM lineage remains unknown. Here we demonstrate detailed XCI analysis in preimplantation embryos and ESCs in clinically relevant nonhuman primate model. We generated heterozygous rhesus monkey (Macaca Mulatta) embryos carrying single nucleotide polymorphisms (SNPs) within transcribed regions of XIST and several other X-linked genes. Allele-specific expression analysis in isolated whole ICMs revealed that XIST and several other analyzed genes are transcribed from both parental alleles suggesting either XaXa or mixture of cells with random XCI. However, detailed bisulfite sequencing analysis indicated that promoters of these genes were not methylated in the ICM while is somatic cells these CpG sites were heavily methylated on one X-chromosome. These results suggest that monkey ICM cells maintain two active X chromosomes. In contrast, majority of monkey ESC lines expressed XIST and other X-linked genes exclusively from one X chromosome suggesting non-random XCI or clonal selection during derivation and culture. ESCs also exhibited methylation of XIST and X-linked genes on one X chromosome similar that seen in somatic cells. These observations are consistent with conclusion that in contrast to pluripotent ICMs, which maintain two active X chromosomes, primate ESCs have already undergone X chromosome inactivation. Moreover, most primate ESC lines display non-random XCI with expression from only one of the parental X chromosomes.

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Poster Board Number: 3598

KDM5B-MEDIATED HISTONE DEMETHYLATION REGULATES EMBRYONIC STEM CELL SELF-RENEWAL AND SAFEGUARDS PRODUCTIVE TRANSCRIPTIONAL ELONGATION

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KDM5B-mediated histone demethylation regulates embryonic stem cell self-renewal and safeguards productive transcriptional elongation ChIP-Seq and RNA-Seq analysis identified that the histone H3 trimethyl lysine 4 (H3K4me3) demethylase KDM5B as a direct downstream target of Oct4 and Nanog in murine and human embryonic stem cells (mESCs). KDM5B expression is highly enriched in the inner cell mass of preimplantation blastocysts and largely restricted to pluripotent cells. Ablation of Oct4 or Nanog markedly reduced KDM5B expression in mESCs. Both loss- and gain-of-function studies have implicated KDM5B as a critical regulator of mESC self-renewal. Knockdown of KDM5B in mESCs displayed compromised proliferation and a predisposition towards differentiation. Forced expression of KDM5B supported clonal expansion of mESC in the absence of LIF. Although KDM5B is believed to function as a promoter-bound repressor, RNA-Seq analysis showed that it paradoxically functions as an activator of a gene network associated with self-renewal. ChIP-Seq revealed that KDM5B is predominantly targeted to intragenic regions and that it is significantly correlated with H3K36me3, a histone modification that marks actively transcribed chromatin. KDM5B is recruited to H3K36me3 via an interaction with the chromodomain protein MRG15 and ChIP-Seq analysis shows high degree of MRG15 colocalization with KDM5B. MRG15 is an ortholog of Eaf3, a component of the yeast Rpd3S complex which functions to repress cryptic intragenic transcription in yeast. In mammals H3K4me3 has been proposed to serve as a nucleation site for the PolII initiation complex. We show that PolII interacts with core subunits of the H3K4 methyltransferase and that inhibition of PolII elongation reduces intragenic H3K4me3 deposition. KDM5B knockdown induced a marked increase in H3K4me3 at intragenic target sites, stimulated loading of PolII, and increased cryptic intragenic transcription. We propose a model in which KDM5B activates a unique self-renewal-associated gene network by repressing intragenic cryptic initiation and maintaining an H3K4me3 gradient important for productive transcriptional elongation.

Poster Board Number: 3600

MAINTENANCE OF GENOMIC STABILITY BY ZSCAN4

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Current advances in tissue stem cells and iPS technologies as potential source for therapies have emphasized the need for methods to stabilize and prevent cancerous transformation of cultured cells. Mouse ES cells retain their genomic integrity following long-term cultures and portray diminutive amounts of chromosomal abnormalities compared to other pluripotent stem cell lines, such as embryonal carcinoma (EC) cells and some human ES cells. However, the mechanism for this characteristic remains to be elucidated. Telomeres are repetitive DNA sequences accompanied by proteins that cap and protect chromosome integrity. Telomere shortening may lead to cancerous transformation through contribution to genomic instability, and has been associated with aging and cellular senescence. By using ES cells we have identified a novel telomere regulation and genomic stabilization mechanism induced by Zscan4 activity. We have further shown that Zscan4 is essential for immortality and long-term culture of mouse ES cells. Although telomerase, the enzyme involved in telomere maintenance, is active in ES cells, we found Zscan4 activates a telomerase independent mechanism for telomere length regulation. Our current study indicates further involvement of Zscan4 in genome stability of mouse ES cells. Zscan4 seems to have a protective effect from DNA damage and moreover, its expression is upregulated, following cells' exposure to genotoxic stress. Taken together, our data reveal a unique mode of genome maintenance in undifferentiated ES cells.

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