CHARLES UNIVERSITY IN PRAGUE
FACULTY OF MEDICINE IN HRADEC KRÁLOVÉ

Mesenchymal Stem Cells: Isolation, Characterization and Potential Clinical Applications

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Doctoral degree programme: **Anatomy, Histology and Embryology**

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Mezenchymové kmenové buňky: Izolace, charakteristika a potenciální klinické aplikace

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Hradec Králové, 2013 defence on: ....

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Author's Declaration

I hereby declare that this dissertation thesis is my own original work and that I indicated by references all used information sources. I also agree with depositing my dissertation in the Medical Library of the Charles University in Prague, Faculty of Medicine in Hradec Králové and with making use of it for study and educational purpose provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

I give my consent to availability of my dissertation's electronic version in the information system of the Charles University in Prague.

Hradec Králové, 14th June, 2014 | Signature of the author

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Abbreviations

ASC	L-ascorbic acid
bFGF	basic fibroblast growth factor
BM	bone marrow
BSA	bovine serum albumine
BSC	biological safety cabinet
CFU-F	colony-forming unit - fibroblast
CSA	catalyzed signal amplification
dif.	differentiation
DMEM	Dulbecco's modified essential medium
DMSO	dimethylsulfoxide
D-PBS	Dulbecco's phosphate buffered saline
DPSCs	dental pulp stem cells
DT	doubling time
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ES	embryonic stem cells
FCS	fetal calf serum
FBS	fetal bovine serum
FGF2	basic fibroblast growth factor
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycan
GEN	gentamycine
GM-CSF	granulocyte macrophage colony stimulating factor
GMP	good manufacturing practice
GvHD	graft versus host disease
HBSS	Hank's balanced salted solution
HE	hematoxylin-eosin
HGF	hepatocyte growth factor
hMSCs	human mesenchymal stem cells
hTERT	human telomerase reverse transcriptase (catalytic unit)
IDO	indoleamine 2,3 dioxygenase
IGF-1	insulin like growth factor 1
IMS	industrial methylated spirits

IPA	isopropyl alcohol
ITS	liquid media supplement (insulin, transferrin, sodium selenite)
MDR1/Mdr1	multidrug resistance protein 1
LIF	leukaemia inhibitory factor
LSAB	labelled streptavidin-biotin
MCP 1	monocyte chemoatractant protein 1
M-SCF	macrophage colony stimulating factor
MSCs	mesenchymal stem cells
neg.	negative
NK	natural killer
No.	number
PCNA	proliferating cell nuclear antigen
PD	population doublings
PDGF-BB	platelet-derived growth factor
PE	phycoerythrin
PEf	plating efficiency
PGE 2	prostaglandin E2
PNC	penicillin
pos.	positive
SCF	stem cell factor
SDF 1	stromal derived factor 1
STM	streptomycin
STRO-1	stromal cell surface marker-1
TGF	transforming growth factor
TGF-β1	transforming growth factor-β1
Treg	regulatory T cells
VEGF	vascular endothelial growth factor
WB	Western blotting

Introduction

As every project, this work started with the blank sheet of paper. Tomas Soukup, pregraduate student of medicine, wanted to become the world famous neurosurgeon and therefore in early 2001 traveled to Hong Kong to learn cultivation techniques and mesenchymal stem cells (MSCs) biology. All this for intervertebral disc regeneration study.

Unfortunately, life has chosen a different path... In 2003 I was confronted with severe autoimmune reaction in my brother's body, in one week he lost all of his Islets of Langerhans and became insulin dependet diabetic. A lot of things changed and focus of this thesis was one of them.

Regenerative medicine

In today's world, science and technology are rapidly developing and we are exploring areas that have never been attempted before. One of the many branches in science is regenerative medicine, which includes stem cells research, which in recent times has become a topic which has generated massive interest globally with every research team trying to make record breaking advances.

Regenerative medicine represents a new paradigm in human healthcare with the potential to resolve unmet medical needs by addressing the underlying causes of disease. The emerging field of regenerative medicine is unique in its aim to augment, repair, replace or regenerate organs and tissue that have been damaged by disease, injury or even the natural aging process. This rapidly evolving, interdisciplinary field is transforming healthcare by translating fundamental science into a variety of regenerative technologies including biologics, chemical compounds, materials and devices. It differs from other fields of medicine in the array of disciplines it brings together and in its ability to create or harness the body's innate healing capacity. Currently, the vast majority of treatments for chronic and/or life-threatening diseases are palliative. Others delay disease progression and the onset of complications associated with the underlying illness. Very few therapies in use today are capable of curing or significantly changing the course of disease. The result is a healthcare system burdened by costly treatments for an aging, increasingly ailing population, with few solutions for containing rising costs. The best way to significantly improve the economics of our current healthcare system is to develop more effective treatments for the most burdensome diseases and conditions - diabetes, neurodegenerative disorders, autoimmune disease, stroke and cardiovascular diseases, for example - to facilitate longer, healthier and more productive lives.

Personally, I am in the field of regenerative medicine for 10 years, and during that time stem cell therapy became a commercial and medical reality. Even though the majority of people perceive

regenerative medicine as something of the future, it is actually here and now. A significant number of regenerative medicine products are already commercially and clinically successful. In addition to over 60,000 stem cell transplants annually performed worldwide for the treatment of oncology and blood-based disorders, it is estimated that in 2012 cell therapy products distributed by biotherapeutic companies generated over $900 million with 160,000 patients receiving treatments. It is widely believed that these numbers are easily doubled when including non-cell-based regenerative medicine products such as scaffolds and other materials.

Analysts suggest there are at least 2,500 ongoing regenerative medicine clinical trials involving tens of thousands of patients for a myriad of clinical indications. An estimated 15 percent of this is industry-sponsored, and the remainder is being sponsored by leading academic centers around the world. Regenerative medicines encompass an array of technologies and therapeutic approaches including cell-based therapies, small molecules and biologics as well as synthetic and bio-based materials designed to augment, repair, replace or regenerate organs and tissues, thereby targeting the root cause of disease. Arguably the most prominent segment of the regenerative medicine industry, the cell therapy sector, is currently engaged in over 1,900 clinical trials around the world (National Institutes of Health, ClinicalTrials, c2005).

Living cells, a pillar of the field, are incorporated into regenerative medicines to achieve a variety of positive effects:

- to replace damaged or diseased cells and/or tissue
- to stimulate an endogenous response that promotes the body's own healing such as an immune response or regeneration in diseased tissue
- to deliver genetic or molecular therapies to targets.

To date, there are approximately 40 cell therapy products commercially distributed in regulated markets (Figure 1.). These cell therapy products encompass variety of cell types including primary cells, progenitor cells, tissue-specific stem cells (adult stem cells), embryonic stem cells and now reprogrammed cells (induced pluripotent stem cells) are in various stages of development. They are being tested for almost every imaginable human condition ranging from large-scale indications like chronic heart failure, cancer and diabetes to orphan indications for which there are few available treatments.

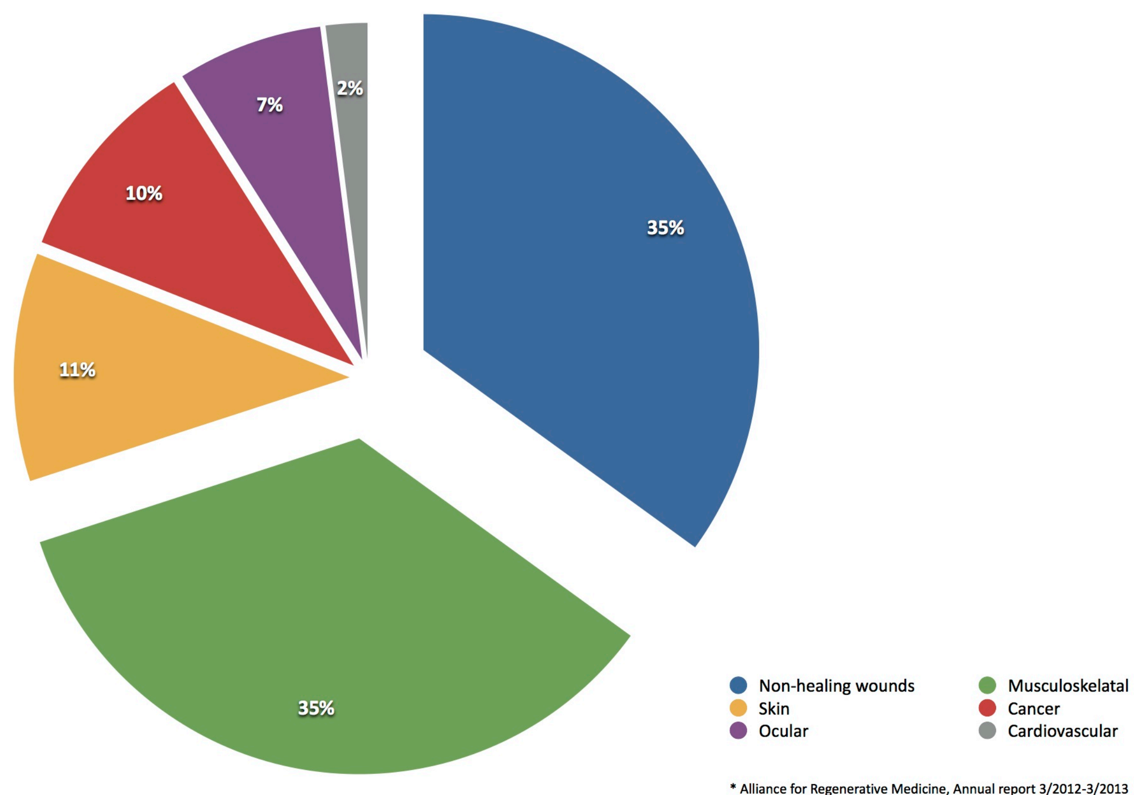


Figure 1. Cell therapy products commercially available - data restricted to countries with formal regulatory frameworks for this type of product, thus excluding cell therapy treatments provided in unregulated markets. Adapted from ARM Annual report 3/2012-3/2013.

Mesenchymal stem cells

The human body consists of about 35 billion cells. To perform diverse functions in a multicellular organism the cells are highly specialized in different tissues. It is estimated that the entire complement of human tissues contains approximately two hundred terminally differentiated cell types. Many cells which are highly specialized have lost the ability to divide and their life-span is shortened. For example enterocytes (intestinal cells specialized for absorption of nutrients) are alive for 4-6 days and red blood cells remain functional for 120 days. Even those differentiated cells that have not lost the ability to divide can enter the cell cycle only 50-60 times (known as the Hayflick's limit). To maintain the structure and function of tissues composed of cells that die or have a limit in their mitotic activity, it is crucial to replenish the pool of lost cells. This task is performed by stem cells (Purstem.eu).

The hMSCs originally described in the 1960s as bone forming cells in the bone marrow (Friedenstein et al., 1966) are rare cells present in the bone marrow with the frequency of one in

10^6 to one in 10^4 mononuclear cells, depending on the age of the patient (Werntz et al., 1996). These cells are able to differentiate into cells of specialized tissues - i.e. chondrocytes (Mackay et al., 1998), myocytes (Prockop, 1997), adipocytes, neural cells (Zhao et al., 2002), beta-pancreatic islet cells (Chen et al., 2004) and lastly osteoblasts (Haynesworth et al., 1992, Jaiswal et al., 1997). The hMSCs were already used in autologous and allogenic stem cell transplantation (Koc et al., 2000, Le Blanc et al., 2004), in treatment of metabolic disorders (Koc et al., 2002), as well as in cardiology (Chen et al., 2004), neurology (Bang et al., 2005) and orthopedic surgery (Kawate et al., 2006). The hMSCs also play an important role in tissue engineering of arteries (Wang et al., 2005), tendon (Sato et al., 2000), cartilage (Ma et al., 2003), lung tissue (Lin et al., 2006), nervous tissue (Yang et al., 2004), and bone (Yao et al., 2005, Wang et al., 2007).

The hMSCs can be easily obtained and expanded from bone marrow in in vitro conditions. However, all protocols published to date have certain disadvantages (Manello et al., 2007). Clinical trials reported thus far (Koc et al., 2000, Le Blanc et al., 2004, Koc et al., 2002, Kawate et al., 2006, Bang et al., 2005, Chen et al., 2004) have used fetal calf serum (FCS) for initial expansion and further propagation of hMSCs. While no serious adverse effects have been reported in these trials, anaphylactic reactions have been reported to other cellular products cultivated with animal serum (Selvaggi et al., 1997). Moreover, use of xenogenic serum carries the potential risk of viral or prion disease transmission. For these reasons, new approaches using human autologous or allogenic plasma or platelet lysates have been pioneered with controversial results.

Current protocols in use require at least one passaging procedure to obtain an adequate number of cells. Requirement for passaging prolongs the period from the bone marrow harvest to the delivery into patient by up to four or six weeks and necessitates replating of cells in an open system or use of bioreactor (Dennis et al., 2007). Moreover some authors reported the loss of multipotentiality and a decrease of osteogenic potential after multiple passagings (Digirolamo et al., 1999, Sugiura et al., 2004).

In 1995, Gronthos and Simmons examined 25 purified recombinant human growth factors for their ability to stimulate growth of CFU-F from purified STRO-1 positive human bone marrow cells. They found that in alpha-MEM with 20% fetal calf serum, supplements such as EGF, PDGF, INF-γ, TNF-α and IL-1 had stimulated growth of hMSCs colonies, while IL-4 and INF-α inhibited them. Simultaneous use of PDGF and EGF increased the size but not the number of CFU-F. Effects of other cytokines, namely M-CSF and FGF-2, were described by Jin-Xiang et al. (2004) and Tsutsumi et al. (2001), respectively. Jin-Xiang et al. (2004) found that M-CSF increased the CFU-F number by 25%. Tsutsumi et al. (2001) found that FGF-2 increased the number of hMSCs when grown in low-

density cultures and that FGF-2 stimulated hMSCs to retain their osteogenic and chondrogenic differentiating ability for a greater number of population doublings.

Serum free culture of adult human bone marrow derived mesenchymal stem cells

The use of MSCs in the clinic will most likely involve more than the minimal manipulation of harvesting and infusion. Expansion and differentiation steps in MSC culture involve the use of bovine serum-containing media. Bovine serum is the most widely used growth supplement for in vitro culture because of its high levels of growth-stimulatory factors and low levels of growth-inhibitory factors. However, use of animal derived products greatly enhances the risk of prion, viral or zoonose contamination. By eliminating our reliance on animal or human based products, the risk of infection is eliminated (Mannello et al., 2007, Halme et al., 2006).

A greater risk than prion or viral infection through fetal bovine serum is the risk of immune reaction. Proteins from serum attach to cells cultured in its presence, acting as antigenic substrates for immune reactions (Tuschong et al., 2002). Selvaggi et al. (1997) showed that human lymphocytes cultured in media containing fetal calf serum resulted in immunogenic reactions when used therapeutically, resulting in an arthus-like reaction. Patients were sensitized to fetal calf proteins carried on the donor cells despite repeated washing of the cells before infusion. Additionally, the detection of newly formed antibodies to fetal calf serum indicated that immune complex formation followed cell transplantation. More recently Chachques et al. (2004) demonstrated that autologous myoblasts cultured in the presence of autologous human serum and transplanted into infarcted hearts decreases the risk of arrhythmia that was identified in previous studies using bovine serum.

An alternative to bovine serum albumin is autologous or allogeneic human serum. Autologous serum has proven to be as effective as bovine serum regarding the isolation and expansion of human MSCs (Stute et al., 2004). Additionally, autologous human serum has been shown to maintain higher cell motility compared to bovine derived serum (Kobayashi et al., 2005) and has eliminated the immune reaction observed with bovine serum cultured cells (Selvaggi et al., 1997 and Chachques et al., 2004). Culturing MSCs in allogeneic human serum results in growth arrest and cell death (Shahdadfar et al., 2005). Microarray experiments demonstrated several regulated genes comparing MSCs cultured in autologous serum as compared to fetal bovine serum. Interestingly, several of these regulated transcripts involve cell cycle and differentiation genes including anti-apoptotic genes and cell cycle genes (Shahdadfar et al., 2005). Alternatively, fresh or frozen human plasma and platelets have been shown to be even more effective than bovine

derived serum in maintaining human MSCs proliferation (Muller et al., 2006). Pytlik et al. (2009) described a simple, yet efficient method of one-step cultivation of large numbers of human multipotent mesenchymal stromal cells. Key components of this method were the co-cultivation of adherent and non-adherent bone marrow cells, the use of special medium supplemented with human pooled serum and the use of additional growth factors and cytokines. However, the use of human serum poses many of the same problems as fetal bovine serum such as variability in composition between donors and the collection of serum from patients with pathology, eventually to be treated with cultured MSCs, making the use of human serum an unattractive option.

The need for a rationally developed serum free medium for the expansion and differentiation of human MSCs is clear for quality control of experiments between laboratories as well as for the culture of cells intended for clinical use. Unfortunately, the use of current serum-free conditions in culturing human MSCs selects for a subpopulation of cells that can survive serum deprivation and continue proliferating (Pochampally et al., 2004). These cells contained longer telomeres than control cells and expressed genes associated with embryonic cells such as octomer-binding transcription factor 4. A new compliment of medium, growth factors, cytokines, etc must therefore be developed specifically to compliment the display of receptors expressed on MSC cell surface ensuring uniform expansion of the MSCs population.

Serum free differentiation of adult human bone marrow derived mesenchymal stem cells

Differentiation of MSCs into osteochondral lineages in serum-free media relies upon the addition of cytokines and/or growth factors (Kuznetsov et al., 1997; Gronthos et al., 1995). Several cytokines and growth factors that promote chondrogenesis have been identified (Heng et al., 2004). For example, various isoforms of the TGF-beta superfamily (Chimal-Monroy et al., 1997), fibroblast growth factor 2 (Quarto et al., 1997) and insulin-like growth factor 1 (Fortier et al., 2002). Furthermore, several non-proteinaceous chemical factors can promote chondrogenesis, such as ascorbic acid (Farquharison et al., 1998), prostaglandin E2 (Miyamoto et al., 2003) and concanavalin A (Mikhailov et al., 1988). The challenge is to find the subtle combination of factors that will induce differentiation down the expected lineage. This is especially difficult in osteochondral differentiation as these tissues (and the supportive growth factors and cytokines) are closely intertwined, dose dependent and temporally dependent. Further, depending upon the dosage and timing of exposure, these supplements will have pleiotropic effects on MSCs.

Serum-free media for the expansion and differentiation of non-human cells, such as embryonic chick mesenchymal cells (Quarto et al., 1997) and rat MSCs (Lennon et al., 1995) have been published using a combination of supplements such as insulin, dexamethasone, FGF-2, PDGF-bb

and EDGF were used to maintain proliferation and differentiation. Significantly less success has been achieved with human cells.

Conflicting results regarding the use of human derived medium supplements have been published. Lin et al. (2005) demonstrated the multipotentiality of human MSCs is maintained when cultured in media containing human plasma. Yamamoto et al. (2003) published similar results that human serum contains factors that not only enable MSC expansion and differentiation, but augments differentiation down the osteogenic and adipogenic lineages (Stute et al., 2004, Oreffo et al., 1997). Alternatively, Shahdadfar et al. (2005) observed that human MSCs grown in autologous human serum proliferated faster than cells cultured in fetal bovine serum; however, they differentiated more slowly than MSCs expanded in fetal bovine serum. Using a microarray, Shahdadfar et al. (2005) demonstrated the differences in gene expression between human and bovine serum cultured cells. MSCs cultured in fetal bovine serum expressed genes indicating they were taken farther along a differentiation pathway as compared to MSCs cultured in human serum. But again, the use of human serum poses many of the same problems as fetal bovine serum such as variability in composition between donors and the collection of serum from patients with pathology, eventually to be treated with cultured MSCs, making the use of human serum an unattractive option. In the end, a serum free medium containing a specific complement of growth factors (at the appropriate times) must be developed to enable MSC differentiation in a GMP setting for clinical application.

Autoimmune disorders

Why does the immune system attack itself and destroy its own tissues? Autoimmune disorders are conditions where the body produces an inappropriate response to healthy tissue and substances normally found in the body, effectively destroying them. The body's immune system is unable to tell the difference between healthy body tissues and antigens such as bacteria, viruses, toxins, cancer cells, blood or tissue from another person. It wrongly identifies the normal tissue as pathogens and attacks its own cells. This leads to the destruction of one or more types of body tissue, abnormal growth of an organ or changes in organ function. These diseases commonly affect the blood vessels, connective tissues, endocrine glands, joints, muscles, red blood cells and skin. Common diseases that are classified into the broad spectrum of autoimmune disorders include multiple sclerosis, type 1 diabetes, lupus, rheumatoid arthritis, uveitis, scleroderma, grave's disease and chronic thyroiditis, among others. There are over 80 types of autoimmune disorders in total. Different diseases are more prevalent in certain races and ethnic groups, and 75% of the patients are female. What causes the inappropriate immune response in many of these disorders

is still unknown, though bacteria and viruses, along with genetic factors, are thought to be involved. These diseases can strike any part of the body with widely varying symptoms, making treatment very difficult. One common form of treatment is immunosuppression, a medication which decreases the immune response in these diseases. However, this treatment also suppresses normal immunity, leaving the body at risk for infection.

These days, developing treatments for autoimmune disorders and inflammation is a significant focus in the regenerative medicine community. The mechanism of action of several of these cell based therapies is still being explored, however, data suggests immunomodulation and suppression can be achieved through cellular interaction between therapeutic cells and the patient's immune system, including anti-inflammatory T-cell responses (Figure 2).

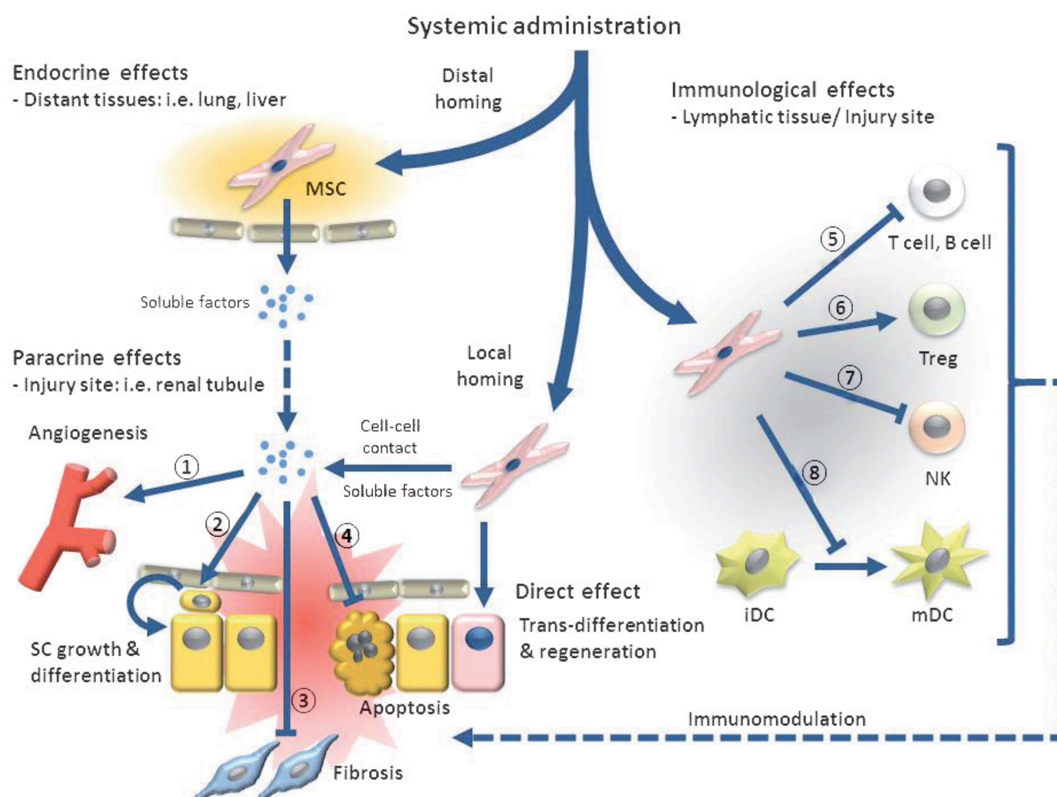


Figure 2. Systemic administration of mesenchymal stem cells can: trigger distal (endocrine) or local (paracrine) effects that include cell mediated actions. 1) Promotion of angiogenesis: vascular endothelial growth factor (VEGF), insulin like growth factor 1 (IGF-1), monocyte chemoatractant protein 1 (MCP 1), basic fibroblast growth factor (bFGF) and interleukin 6 (IL 6). 2) Stem cell growth and differentiation: stem cell factor (SCF), leukemia inhibitory factor (LIF), macrophage colony stimulating factor (M-CSF), stromal derived factor 1 (SDF 1), angiopoietin 1 and activin A. 3) Inhibition of fibrosis: hepatocyte growth factor (HGF), bFGF, adrenomedullin. 4) Inhibition of apoptosis: VEGF, HGF, IGF 1, transforming growth factor (TGF) β, bFGF, granulocyte macrophage colony stimulating factor (GM-CSF), activin A and thrombospondin 1. Immune mediated effects include the following (5 to 8). 5) Suppression of T and B cells: human leukocyte antigen G5, HGF, inducible nitric oxide synthase, indoleamine 2,3 dioxygenase (IDO), prostaglandin E2 (PGE 2), bFGF and TGF β. 6) Induction of regulatory T cells (Treg) differentiation and expansion by TGF β expression. 7) Inhibition of natural killer (NK) cells by secretion of IDO, PGE 2 and TGF β. 8) Inhibition of dendritic cell maturation by secretion of PGE 2.
Figure reproduced from Carrión and Figueroa, 2011.

Several of these technologies are based on mesenchymal stem and progenitor cell populations derived from a variety of adult tissue sources. Here I am presenting the list of leading biotech companies involved in the autoimmune disordes treatment.

Athersys, Inc. is testing its lead product, MultiStem (allogeneic adult stem cell technology), for prevention or reduction of GvHD in cases of patients undergoing allogeneic hematopoietic stem cell transplants for the treatment of leukemia and related conditions. Clinical studies yielded positive results in the Phase 1 trial, potentially increasing treatment and prevention options for this autoimmune disease. Additionally, Athersys is conducting a Phase 2 clinical study with partner Pfizer to test the safety and efficacy of MultiStem in individuals suffering from Inflammatory Bowel Disease.

Celgene is commercializing placenta-derived stem cell therapies for autoimmune and inflammatory diseases. They are currently conducting several clinical studies of their product PDA-001 in autoimmune and inflammatory conditions including Phase 2 studies in both Crohn's disease and rheumatoid arthritis and Phase 1 studies in multiple sclerosis and sarcoidosis.

NeoStem is developing a T-cell therapeutic, Athelos, which works to restore immune balance in GvHD patients by enhancing T-regulatory cell numbers and function. They are currently in a Phase 1 trial.

Osiris's lead product, Prochymal is in Phase 3 studies in the United States for GvHD, and has been approved in Canada. The product demonstrated significant survival benefits in patients with the most severe forms of GvHD. Osiris is also testing Prochymal in Phase 2 studies for Crohn's disease.

Tigenix , a leading European cell therapy company, is making several advancements with their adult stem cell programs targeting autoimmune and inflammatory diseases. Their stem cell platform uses allogeneic adipose tissue derived expanded stem cells. Tigenix currently has two products in clinical studies for autoimmune and inflammatory disorders, Cx601 in Phase 3 development for Crohn's disease and Cx611 in Phase 2 studies for rheumatoid arthritis.

Therapeutic use of MSCs in autoimmune and inflammatory diseases

Given their vast proliferative potential, extensive immunosuppressive properties, and also the ease of access to proper tissue sources, therapies with autologous or allogenic MSCs have been tested in a variety of immunemediated disease models, including experimental allergic encephalomyelitis - a model of multiple sclerosis (Rafaei et al., 2009, Bai et al., 2009), diabetic NOD/SCID mice (Lee et al., 2006) and collagen-induced arthritis (Augello et al., 2007, Gonzales et al., 2009). Results have

been encouraging, but not altogether consistent, particularly in the case of arthritis (Schurgers et al., 2010).

Moreover, at the time of writing this thesis, 329 registered human trials on MSCs were found at the National Institutes of Health, c2005 ClinicalTrials.gov website, including 28 for graft versus host disease (GvHD), 137 for diabetes, 19 for Crohn's disease or ulcerative colitis and 26 for multiple sclerosis. Some of these trials point to non-immune-mediated conditions that are associated with tissue injury, such as hepatic cirrhosis, myocardial infarction or congestive heart failure. In several instances it has become apparent that MSCs are not necessarily replacing diseased tissues or differentiating into separate cell lineages, but seem to exert a complex pattern of trophic, regenerative and antiinflammatory effects (Block et al., 2009, Chen et al., 2008). In humans, the most studied application for MSCs is GvHD, a complication of hematopoietic stem cell transplantation in which donor T cells attack an immunocompromised and genetically disparate recipient (English et al., 2010). In 2004, Le Blanc and colleagues treated a 9-year-old boy with severe treatment-resistant acute GvHD of the gut and liver with third party haplo-identical motherderived MSCs. Clinical response was striking, with improvement of liver and intestinal function. The most recent placebo controlled trials confirmed the significant improvement in liver and gastrointestinal GvHD, but did not reach significance for durable complete responses or other primary endpoints (Osiris, Prochymal, Phase 3).

Objectives

From my early scientific steps I tried to constantly challenge conventional wisdom, to take the contrarian route and to create novel solutions. Following my first experiments with low-FCS containing media and experimental work with dental pulp stem cells I was invited to be a part of the collaborative project - PurStem. As a team leader I was personally responsible for the work package focused on serum-free isolation, growth and differentiation of MSCs. The objectives summarized in this chapter are in relation to this project work package.

Stem cells offer a promising avenue to therapy for a wide range of human diseases. However, for this potential to be realized, a consistent and plentiful supply of well-characterised stem cells is essential. To date, there has been relatively little progress in the development of new culture technologies for the large-scale manufacture of mesenchymal stem cells. The current state-of-the-art has several weaknesses, in that, there are no standards for the characterisation, isolation or identification of MSCs from any tissue, nor are there standard protocols for differentiation of MSCs to various lineages. Additionally, surface markers used for MSC characterisation lack specificity and cryopreservation protocols are not standardised. Critically, current production methods for MSC require the use of animal products with major contaminant implications.

The objectives of this thesis were to:

- Examine existing methods and approaches to the preparation of MSCs
- Identify current best practice
- Standardise the technology by means of a unified operating protocol
- Develop new methods of culture and new media formulations by identifying the repertoire of growth factor receptors that exists on the surface of the cell
- Use this information to develop new media using a combinatorial approach to the selection of growth factor supplements to modulate and optimise the growth kinetics and differentiation of the cells
- Utilise recombinant human growth factors and so benefit from freedom of reliance on serum
- Generate a validated standard operating procedure for serum-free MSCs culture

Methods & SOPs

For this study, bone marrow was obtained from 8 consecutive patients (healthy donors) undergoing total hip replacement where 10-15ml of BM were aspirated. Moreover 18 reference DPSCs lines from impacted third molars obtained from healthy donors were isolated. Donors or their legitimate representative were supposed to subscribe informed consent according to guidelines of Ethical committee of the Medical Faculty in Hradec Králové.

For the validation purposes and for creation of collaborative standardized procedures, 4 bone marrow samples were bought and distributed (AllCells, CA).

Processing of BM was performed as described in following Standard Operating Protocol, processing of dental pulp was performed as described previusly by Suchánek et al., 2009 and a pre-screen of factor combinations implemented using passaged MSCs grown in serum or reduced serum (Soukup et al., 2006, Karbanova et al., 2011) and the directly isolated in vivo MSC that had not been exposed to serum.

Following protocols, I had created, served as a uniform method of MSC isolation, culture and cryostorage procedures in each PurStem partner laboratory, resulting in comparability in culture protocols, differentiation protocols and cell characterization by differentiation and FACS. Outlined here are the protocols agreed upon by PurStem partners in the beginning of the PurStem project.

These protocols are separated into three sections:

A) MSCs isolation protocol

B) MSCs subculture protocol

C) MSCs cryopreservation protocol and Preparation of cryopreservation medium

A) MSCs isolation protocol

I. MATERIALS AND EQUIPMENT

Solutions

1. Human bone marrow sample
2. Complete hMSCs medium
3. Dulbecco's phosphate buffered saline (D-PBS)
4. 4.0% (v/v) acetic acid
5. 70% isopropyl alcohol (IPA) or industrial methylated spirits (IMS)
6. 0.25% trypsin-1mM ethylenediaminetetraacetic acid (EDTA)

Materials

1. Sterile serological pipettes (25ml, 10ml, 5ml)

2. Sterile pipette tips (P100 & P1000)
3. 1.5 ml snap-top microfuge tubes
4. 50 ml centrifuge tubes
5. Polystyrene canted neck 175 cm^2 flasks (T-175), with vented cap or equivalent
6. Disposable container for liquid waste

Equipment

1. Biological safety cabinet class II (BSC)
2. Incubator, (37^oC, 5% CO_2, 90% humidity)
3. Centrifuge
4. Haemocytometer with cover slip
5. Z2 Counter, ViCell analyser
6. Inverted light microscope
7. 4^oC refrigerator
8. Gilson pipettes (P100 & P1000)
9. Pipette filter
10. Calculator

II. SOLUTIONS

1. Basal medium is warmed to 37 ^oC using a water bath
2. Basal medium is alpha MEM with Glutamax (Invitrogen, USA, cat. No. 32561-029)
3. 10% of total end volume of basal medium is removed
4. 10% of total end volume is mixed with fetal bovine serum (PAA, Austria, cat. No. A11-532 and A15-532, lot No. A53206-0618)
5. 100 units/ml of penicillin (PNC) and streptomycin (STM) are added
6. Medium is stored at 4 ^oC until use
7. 1 ng/ml FGF2 is added (PeproTech, USA, cat. No. 100-18B) immediately before using media

III. COLLECTION OF HUMAN BONE MARROW

Human bone marrow is either aspirated or purchased, then aliquoted and sent to the laboratory. Sodium heparin is used as the anti coagulant for the aspiration.

Donors are treated in accordance with the local donor programme and in full compliance with ethical approval.

IV. hMSCs ISOLATION PROCEDURE

hMSCs are isolated by direct plating (i.e. plastic adherence) without selection methods such as MACS or MNC isolation.

Isolation Day 0

1. The interior work area of the BSC is wiped with alcohol.
2. D-PBS and complete hMSC media are transfered to the BSC.

3. Human bone marrow sample is transfered to the interior workspace of the clean and operating BSC.
4. Human bone marrow aspirate is carefully transfered from the transportation tubes into a sterile 50 ml centrifuge tube. Total volume of the sample is measured with a sterile serological pipette.
5. Total bone marrow aspirate volume is aliquoted into several 50 ml centrifuge tubes and diluted 1:1 with D-PBS for the initial wash. The suspension is mixed by inverting the tube several times gently.
6. Tubes are centrifuged at 900 g (2000rpm) for 10 minutes at room temperature.
7. Tubes are carefully removed from the centrifuge and carefully placed back into the BSC as the cell pellets are very soft and can be easily disturbed.
8. Using a sterile serological 25 ml pipette, as much of the supernatant as possible is carefully removed without disturbing the cell pellets.
9. hMSCs are resuspended again in 40 ml of D-PBS.
10. Pellets are then combined in a fresh sterile 50 ml tube. If multiple tubes have been used, the tubes are rinsed with a total volume of 5 ml D-PBS and this wash is added to the combined pellets. Total volume of cell suspension is measured with a sterile serological pipette.
11. 50 μl aliquot of the bone marrow suspension is removed for cell counting using a P100 pipette, and added to 450 μl of D-PBS in a microfuge tube. Suspension is then mixed by closing the lid tightly and flicking with finger once or several times until mixed well.
12. 50 μl of 4% (v/v) acetic acid is pipetted into a microfuge tube.
13. From the bone marrow/D-PBS suspension, a 50 μl aliquot is removed and added to the 4% (v/v) acetic acid in the microfuge tube. Suspension is mixed and let sit for at least one minute so that the erythrocytes have sufficient time to lyse from exposure to the acetic acid.
14. Suspension is pipetted onto a haemacytometer. Number of unlysed cells is counted. The cell number/ml is calculated by multiplying the number of cells counted in the 25 square grid by the dilution factor of 20 and then multiplying by 10^4. The total cell number is counted by multiplying the cell number/ml by the total suspension volume.
15. Cell number and viability are then verified by Z2 Counter (Beckman Coulter, USA) and ViCell analyser (Beckman Coulter, USA).

16. Primary hMSC are plated at a cell density 25,000 cells/cm^2. The number of T-175 flasks required for the number of cells counted is set. The volume of complete hMSC medium necessary to be added to the cell suspension is counted, so that there is enough volume to allow 5ml of the cell suspension to be pipetted into each flask. This volume is added to the cell suspension.
17. The volume of complete hMSC medium calculated earlier is added to the cell suspension.
18. 25 ml of complete hMSC medium is added into each T-175 flask evenly such that the cell suspension is evenly distributed in the flask. Volumes are adjusted appropriately when using different sized flasks.
19. 5 ml of the cell suspension is added into each flask. The cell suspension have to be spread evenly through the flask.
20. The flasks of cells lying horizontally are placed in an incubator at 37^oC, 5% CO_2, 90% humidity.

Day 4

1. Interior workspace of the BSC is wiped down with alcohol.
2. All solutions are transfered to the BSC.
3. Cell culture flasks are removed from the incubator.
4. The outside of the flasks is wipe down with alcohol and transfered to the BSC.
5. Flasks are gently swirled to dislodge the red blood cells and other unattached cells.
6. Media containing the unattached cells is removed using a sterile serological pipette.
7. 10ml D-PBS is added to each flask, flasks are swirled to suspend the unattached cells, and D-PBS is aspirated without touching the monolayer.
8. 35ml of fresh complete hMSC medium with FGF2 is added.
9. Individual attached cells are observed under the inverted light microscope at 4x magnification.
10. Flasks are finally returned to the incubator.

Subsequent feeding of cultured hMSCs

1. Note: hMSCs cultures are to be fed twice a week.
2. Interior workspace of the BSC is wiped down with alcohol.
3. All solutions are transfered to the BSC.
4. Cell culture flasks are removed from the incubator.

5. The outside of the flasks is wipe down with alcohol and transfered to the BSC.
6. Conditioned medium is removed from the flask by aspiration with a sterile aspiration pipette.
7. 35ml of warm, fresh complete hMSC medium with FGF2 is added.
8. Attached cells are observed under the inverted light microscope at 4x magnification.
9. Flasks are returned to the incubator.

B) MSCs subculture protocol

I. MATERIALS AND EQUIPMENT

Solutions

1. 0.25% Trypsin-1mM EDTA
2. Complete hMSC medium
3. Dulbecco's phosphate buffered saline (D-PBS)
4. Flasks of hMSC ready for subculture
5. Isopropyl alcohol (IPA)

Materials

1. 175cm^2 polystyrene tissue culture flasks with vented caps (T-175)
2. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
3. Sterile filter pipette tips (for P100)
4. Sterile 15 ml centrifuge tubes
5. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Timer
7. Haemocytometer with cover slip
8. Z2 Counter, ViCell analyser
9. Gilson pipette (P100)
10. Pipette filler

II. PROCEDURE

Note: The primary MSCs culture should be passaged when discrete colonies of MSCs have formed, but before multilayer growth occurs. In subsequent passages, MSCs should be subcultured at 80-90% confluence.

1. A few hours before beginning the procedure, 0.25% Trypsin-1mM EDTA is removed from the freezer, complete hMSC medium from the 4ºC refrigerator and D-PBS from room temperature storage and are allowed to come to room temperature.
2. The BSC is turned on and the interior work area is wiped down with alcohol.
3. Wiped containers with solutions at ambient temperature for a minimum of one and a half hours are placed in BSC.
4. Flasks with hMSC are removed from the incubator, wiped down with alcohol and placed in the BSC.
5. Medium from each flask is aspirated using a 25 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
6. 10 ml D-PBS is added to each T-175 flask using a 10 ml sterile serological pipette to wash the hMSC. (volumes are adjusted according to the volume of culture flasks)
7. D-PBS is aspirated using a 10 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
8. 5 ml 0.25% Trypsin – 1mM EDTA is added to each flask using a 5ml sterile serological pipette. (volumes are adjusted according to the volume of culture flasks)
9. Flasks are incubated at room temperature for 5-8 minutes.
10. Cells are observed using an inverted light microscope to ensure that the hMSC have dislodged from the surface of the flask. If they are still adherent, flasks are incubated at room temperature until the hMSC are in suspension. Flasks are tapped sharply with gloved hand to ensure detachment.
11. The length of incubation time of hMSC in 0.25% Trypsin-1mM EDTA is recorded.
12. 0.25% Trypsin – 1mM EDTA is inactivated by adding an equal volume of prepared complete hMSC medium without FGF2 to each flask using a sterile serological pipette.
13. The suspension is pipetted over the bottom surface of the vessel several times to fully dislodge the hMSC, and then the cell suspension is transfered to a sterile 15 ml centrifuge tube. Suspension is centrifuged for 10 minutes at 900 g.
14. The interior work area of the BSC is wiped down with alcohol.
15. Centrifuge tubes are transfered from the centrifuge to the BSC. The supernatant is aspirated off and discarded in the container of bleach.
16. Cell pellets are resuspended and pooled from each centrifuge tube in a known volume of complete hMSC medium with FGF2 (5-10 ml). The total volume of the final hMSC suspension is measured.
17. 50 µl aliquot of the hMSC cell suspension is removed for cell counting.

18. Cell number/ml is calculated by multiplying the number of cells counted in the 25 square grid by 10^4. Total cell number is calculated by multiplying the cell number/ml by the total volume of the cell suspension.
19. 4,500 cells/cm^2 are plated in 35 ml of prepared complete hMSC medium with FGF2 in each T-175 flask. The flasks of cells lying horizontally are placed in an incubator at 37°C, 5% CO_2, 90% humidity. (Note: Ensure the cells are evenly distributed in the flask by gently rocking.)
20. hMSC are fed twice per week and observed under an inverted light microscope before they are fed.
21. Note: When hMSC have formed a semi-confluent monolayer, decide whether to subculture the hMSC again, or cryopreserve.

C) MSCs cryopreservation protocol

I. MATERIALS AND EQUIPMENT

Solutions

1. Flasks of hMSC ready for cryopreservation
2. Complete hMSC medium
3. 0.25% trypsin – 1mM EDTA
4. hMSC freezing medium
5. FBS (PAA, Austria, cat. No. A11-532 and A15-532, lot No. A53206-0618)
6. DMSO (Sigma-Aldrich, USA)
7. Isopropyl alcohol (IPA)

Materials

1. Serological pipettes (25 ml, 10 ml, 5 ml)
2. Sterile pipette tips (for P100)
3. Sterile 50ml tube
4. Sterile 15 ml centrifuge tubes
5. Sterile 1ml cryovials
6. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Timer
7. Haemocytometer with cover slip
8. Z2 Counter, ViCell analyser
9. Mr Frosty

10. -80°C freezer
11. Gilson pipettes (P100 & P1000)
12. Pipette filler
13. Liquid nitrogen freezer

II. PROCEDURE

1. A few hours before beginning the procedure, 0.25% Trypsin-1mM EDTA is removed from the freezer, complete hMSC medium from the 4°C refrigerator and D-PBS from room temperature storage and are allowed to come to room temperature.
2. The BSC is turned on and the interior work area is wiped down with alcohol.
3. Wiped containers with solutions at ambient temperature for a minimum of one and a half hours are placed in BSC.
4. Flasks with hMSC are removed from the incubator, wiped down with alcohol and placed in the BSC.
5. Medium from each flask is aspirated using a 25 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
6. 10 ml D-PBS is added to each T-175 flask using a 10 ml sterile serological pipette to wash the hMSC. (volumes are adjusted according to the volume of culture flasks)
7. D-PBS is aspirated using a 10 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
8. 5 ml 0.25% Trypsin – 1mM EDTA is added to each flask using a 5ml sterile serological pipette. (volumes are adjusted according to the volume of culture flasks)
9. Flasks are incubated at room temperature for 5-8 minutes.
10. Cells are observed using an inverted light microscope to ensure that the hMSC have dislodged from the surface of the flask. If they are still adherent, flasks are incubated at room temperature until the hMSC are in suspension. Flasks are tapped sharply with gloved hand to ensure detachment.
11. The length of incubation time of hMSC in 0.25% Trypsin-1mM EDTA is recorded.
12. 0.25% Trypsin – 1mM EDTA is inactivated by adding an equal volume of prepared complete hMSC medium without FGF2 to each flask using a sterile serological pipette.
13. The suspension is pipetted over the bottom surface of the vessel several times to fully dislodge the hMSC, and then the cell suspension is transfered to a sterile 15 ml centrifuge tube. Suspension is centrifuged for 10 minutes at 900 g.
14. The interior work area of the BSC is wiped down with alcohol.

15. Centrifuge tubes are transfered from the centrifuge to the BSC. The supernatant is aspirated off and discarded in the container of bleach.
16. Cell pellets are resuspended and pooled from each centrifuge tube in a known volume of complete hMSC medium with FGF2 (5-10 ml). The total volume of the final hMSC suspension is measured.
17. 50 µl aliquot of the hMSC cell suspension is removed for cell counting.
18. Cell number/ml is calculated by multiplying the number of cells counted in the 25 square grid by 10^4. Total cell number is calculated by multiplying the cell number/ml by the total volume of the cell suspension.
19. Cell suspension is centrifuged for 10 minutes at 900 g at room temperature.
20. Centrifuge tubes are transfered from the centrifuge to the BSC. The supernatant is aspirated off and discarded in the container of bleach.
21. Cell pellet is resuspended in an appropriate volume of freezing medium resulting in $1x10^6$ cells/ml.
22. The cell suspension is aliqoted into appropriately labeled cryovials.
23. Note: Ensure cryovials are labeled with the following - cell batch No., No. of cells/ vial, passage No., date, operators initials or name
24. Cryovials are placed into Mr. Frosty.
25. Mr Frosty is transfered to -80°C freezer and left there overnight.
26. After cryovials have been at -80°C freezer overnight, they are transfered to liquid nitrogen storage container.
27. Note: Record rack, box and position within box that the cryovials are placed.

III. PREPARATION OF CRYOPRESERVATION MEDIUM

1. A few hours before beginning the procedure, an aliquot of FBS is removed from the freezer, an aliquot DMSO from room temperature storage.
2. BSC is turned on and wiped down with alcohol.
3. Note: Ensure solutions have been at ambient temperature for a minimum of one and a half hours.
4. Outside of all containers is wiped and solutions are placed in BSC.
5. 45ml of FBS are transfered to the sterile 50ml tube using a serological pipette.
6. 5ml of DMSO are added to the FBS to make a final volume of 50ml of freezing medium (90% FBS, 10% DMSO).
7. The tube is labeled with ‘Cryopreservation media’ and date.

8. Media is stored at 4°C until required for cryopreservation.

To evaluate CFU-F frequency (CFU-F assay), 100 µl volume of the marrow suspension were plated in $75cm^2$ Petri dishes (TPP, Switzerland). The medium was changed after 72 hours from the first plating and then twice a week. After 2 weeks of primary culture, cells were washed with D-PBS, fixed with 3.7% formaldehyde in D-PBS, stained with 1% methylene blue in borate buffer (10 mM, pH 8.8) for 30 minutes, then washed with distilled water and the colonies were counted. All determinations were performed in duplicate and expressed as mean values. CFU-F frequency was used to calculate the population doublings of first-confluence cultures.

Within P0, P1 and P3 DNA analysis and phenotype were assessed using propidium iodide staining and flow cytometry (Cell Lab Quanta, Beckman Coulter, USA). For phenotypic analysis, cells were detached and stained sequentially with immunofluorescent primary antibodies:

Antibody	Fluorochrome	Clone	Supplier
anti-CD105	FITC	MEM-226	BioLegend
anti-CD105	AF488	SN6	Caltag
anti-CD105	PE	SN6	Caltag
anti-CD105	PE	SN6	Serotec
anti-CD105	PE	1G11	Beckman Coulter
anti-CD106	PE	STA	Biolegend
anti-CD106	PE	1.G11B1	Chemicon
anti-CD106	PE	51-10C9	BD Biosciences
anti-CD117	PE	104D2	Caltag
anti-CD117	PE	YB5.B8	eBioscience
anti-CD119	PE	GIR-208	eBioscience
anti-CD13	FITC	WM15	Serotec
anti-CD133	PE	EMK08	eBioscience
anti-CD133	PE	293C3	Miltenyi Biotec
anti-CD14	PE	M5E2	BD Biosciences
anti-CD14	FITC	RMO52	Beckman Coulter
anti-CD140a	PE	16A1	BioLegend

anti-CD140b	PE	18A2	BioLegend
anti-CD146	AF488	P1H12	Millipore
anti-CD146	PE	P1H12	Chemicon
anti-CD146	PE	TEA1/34	Beckman
anti-CD146	PE	P1H12	BD Biosciences
anti-CD166	PE	3A6	Beckman
anti-CD166	PE	3A6	BD Biosciences
anti-CD18	PE	7.00E+04	Beckman
anti-CD19	FITC	HIB19	BD Biosciences
anti-CD19	FITC	J4.119	Beckman Coulter
anti-CD19	PE	J4.119	Beckman Coulter
anti-CD197	FITC	3D12	eBioscience
anti-CD221	PE	1H7	Lifespan
anti-CD222	FITC	MEM-238	Biolegend
anti-CD222	FITC	MEM-238	Exbio
anti-CD235a	FITC	JC159	Dako
anti-CD235a	PE	JC159	Dako
anti-CD271	PE	C40-1457	BD Biosciences
anti-CD271	PE	ME20.4-1.H4	Miltenyi Biotec
anti-CD271	APC	ME20.4-1.H4	Miltenyi Biotec
anti-CD271	FITC	ME20.4	eBioscience
anti-CD29	AF488	TS2/16	BioLegend
anti-CD29	FITC	TDM29	Millipore
anti-CD29	PE	TS2/16	BioLegend
anti-CD29	PE	MEM-101A	Caltag
anti-CD29	PE-Cy5	TS2/16	BioLegend
anti-CD309	PerCP/Cy5.5	HKDR-1	BioLegend
anti-CD31	PE	WM59	BioLegend
anti-CD31	PE	MBC 78.2	Invitrogen

anti-CD31	FITC	WM59	Serotec
anti-CD33	FITC	HIM3-4	BD Biosciences
anti-CD33	PE	D3HL60.251	Beckman Coulter
anti-CD34	FITC	4H11	Biolegend
anti-CD34	FITC	581 (Class III)	Invitrogen
anti-CD34	PE	581	Beckman
anti-CD34	PE	581 (Class III)	Caltag
anti-CD34	PE	563 (class III)	BD Biosciences
anti-CD34	PE	BIRMA-K3 (class III)	Dako
anti-CD34	PerCp	8G12 (class II)	BD Biosciences
anti-CD34	FITC	581	Beckman Coulter
anti-CD34	PE	QBEnd10	Beckman Coulter
anti-CD44	FITC	MEM-85	Caltag
anti-CD44	FITC	SFF-2	Bender
anti-CD44	PE	IM7	BioLegend
anti-CD44	PE	MEM-85	Invitrogen
anti-CD44	PE-Cy5	IM7	BioLegend
anti-CD45	FITC	HI30	Caltag
anti-CD45	FITC	T29/33	Dako
anti-CD45	PE	HI30	BD Biosciences
anti-CD45	Pe-Cy7	HI30	BD Biosciences
anti-CD45	FITC	J.33	Beckman Coulter
anti-CD45	PE	J.33	Beckman Coulter
anti-CD49d	FITC	HP2/1	Beckman
anti-CD49e	FITC	SAM-1	Beckman
anti-CD49e	FITC	SAM-1	Chemicon
anti-CD63	FITC	CLBGran/12	Beckman
anti-CD63	FITC	CLBGran/12	Caltag
anti-CD63	PE	CLBGran/12	Beckman Coulter

anti-CD71	FITC	MEM-75	BioLegend
anti-CD71	FITC	T56/14	Caltag
anti-CD71	FITC	YDJ1.2.2	Beckman Coulter
anti-CD73	PE	AD2	BD Pharmingen
anti-CD73	PE	AD2	BD Biosciences
anti-CD81	FITC	JS-81	BD Biosciences
anti-CD90	FITC	F15-42-1	Chemicon
anti-CD90	FITC	F15-42-1-5	Beckman
anti-CD90	PE	F15421	Serotec
anti-CXCR4	PE	12G5	Caltag
anti-HLA I	FITC	Tü149	Caltag
anti-HLA-DR	PE	Tü36	Caltag
anti-HLA-DR	PE	Immu-357	Beckman Coulter
anti-HLA-DR	FITC	L243	BD Biosciences
anti-OCT3/4	PE	EM92	eBioscience

Percentage of positive cells was determined as percentage of cells with higher fluorescence intensity than the upper 0.5% isotype immunoglobulin control. Classification criteria: <10% no expression, 11-40% low expression, 41-70% moderate expression and >71% high expression.

For karyotyping cells (subcultured at a 1:3 dilution, both early passages and after reaching Hayflick´s limit) were after 24 hours cultivation subjected to a 4-hour Demecolcemid (Sigma-Aldrich, USA) incubation followed by trypsin-EDTA detachment and lysis with hypotonic KCl and fixation in acid/alcohol. Metaphases were analyzed after GTG banding using software Ikaros v5.0 (MetaSystems, USA).

For detection of cytoskeletal proteins, cells were fixed with methanol at -20°C for 2 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. For other intracellular molecules, cells were fixed with 4% paraformaldehyde at 20°C for 10 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. For cell surface antigens, cells were fixed with 4% paraformaldehyde at 20°C for 10 minutes. Blocking and diluent solution consisted of phosphate-buffer saline (D-PBS), 1% BSA, and 1% serum (Sigma-Aldrich, USA) from the same species as was the primary antibody. Slides were blocked for 30 minutes, incubated sequentially for 30 minutes each with primary antibodies and

fluorescein- or phycoerythrin-coupled anti-mouse or anti-rabbit IgG antibody. Between each step, slides were washed with PBS containing 0.3% BSA.

Three in vitro characterisation assays were also conducted, namely, adipogenesis, chondrogenesis and osteogenesis assays. Each assay was conducted once with 3 replicate wells.

Osteogenic differentiation of hMSCs

I. MATERIALS AND EQUIPMENT

Solutions

1. Complete hMSCs medium
2. Fetal bovine serum (PAA, Austria, cat. No. A11-532 and A15-532, lot No. A53206-0618)
3. DMEM low-glucose (Sigma-Aldrich, USA)
4. Dexamethosone 1mM stock (Sigma-Aldrich, USA)
5. Ascorbic acid 2-Phosphate 10µM (Sigma-Aldrich, USA)
6. β Glygerolphosphate (Sigma-Aldrich, USA)
7. Penecillin/Streptomycin (Invitrogen, USA)
8. 0.25% Trypsin-1mM EDTA
9. Dulbecco's phosphate buffered saline (D-PBS)
10. Flasks of hMSC ready for subculture
11. Isopropyl alcohol (IPA)

Materials

1. 6 well cell culture plates
2. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
3. Sterile filter pipette tips
4. Sterile 15 ml centrifuge tubes
5. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Timer
7. Haemocytometer with cover slip
8. Gilson pipettes (P10, P200 and P1000)
9. Pipette filler

II. PROCEDURE

1. Osteogenic media is prepared according to this table:

Reagent	Volume (to make 100 ml)	Final Concentration
DMEM low-glucose	87.5 ml	
Dexamethasone 1 mM	10 µl	100 nM
Ascorbic acid 2-P 10 mM	0.5 ml	50 µM
β Glygerolphosphate 1 M	1 ml	10 mM
FBS	10 ml	10%
PNC/STM	1 ml	100 U/ml PNC, 100 µg/ml STM

2. Cells are trypsinized, neutralised and counted.
3. MSCs are seeded $3x10^4$ cells / well.
4. 3 control wells and 3 osteogenic test wells are set up and marked.
5. 2 ml of complete medium and osteogenic medium are added per well.
6. Plates are placed in an incubator at 37^oC, 5% CO_2, 90% humidity.
7. After 24 hours cells should have adhered to the surface of the plate.
8. Medium is changed twice a week, ensuring the correct medium is added to each well.
9. Assay is harvested between 10 and 17 days depending on the condition of the monolayer. Cells need to be harvested before they peel as they will be stained.
10. 1 control and 1 test well is stained with fast violet combined with von Kossa staining.
11. Remaining 2 wells for each are used for Alizarin red - calcium quantification.

Chondrogenic differentiation of hMSCs

This protocol describes the processes for the chondrogenic differentiation of hMSC using a pellet culture system.

I. MATERIALS AND EQUIPMENT

Solutions

1. Complete hMSCs medium
2. DMEM high-glucose (Sigma-Aldrich, USA)
3. Dexamethosone 1mM stock (Sigma-Aldrich, USA)
4. Ascorbic acid 2-Phosphate 5 mg/ml (Sigma-Aldrich, USA)
5. L-Proline 4 mg/ml (Sigma-Aldrich, USA)
6. ITS supplement (Sigma-Aldrich, USA)
7. TGF-β3 20 µg/ml
8. Penecillin/Streptomycin (Invitrogen, USA)

9. 0.25% Trypsin-1mM EDTA
10. Dulbecco's phosphate buffered saline (D-PBS)
11. Flasks of hMSC ready for subculture
12. Isopropyl alcohol (IPA)

Materials

1. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
2. Sterile filter siliconized pipette tips
3. Sterile 15 ml centrifuge tubes
4. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Timer
6. Haemocytometer with cover slip
7. Gilson pipettes (P10, P200 and P1000)
8. Pipette filler

II. PROCEDURE

1. Incomplete chondrogenic media is prepared according to this table:

Reagent	Volume (to make 100 ml)	Final Concentration
DMEM high-glucose	95 ml	
Dexamethasone 1 mM	10 µl	100 nM
Ascorbic acid 2-P 5 mg/ml	1 ml	50 µg/ml
L-Proline 4 mg/ml	1 ml	40 µg/ml
ITS supplement	1ml	
Sodium pyruvate	1 ml	1 mM
PNC/STM	1ml	100 U/ml PNC, 100 µg/ml STM

2. To 1 ml of incomplete chondrogenic media 0.5µl of TGFβ-3 is added to give a final concentration of 10 ng/ml. This is complete chondrogenic medium.
3. Cells are trypsinized, neutralised and counted.
4. $2.5x10^5$ MSCs are needed / pellet culture.
5. 3 pellet cultures are set up to be harvested on day 21.

6. Required number of cells for all the pellet cultures are placed into a tube and centrifuged at 100g for 5 min.
7. Cells are resuspended in complete chondrogenic medium (0.5 ml per each chondrogenic pellet).
8. Tubes are centrifuged at 100g for 5 min.
9. The tubes are transfered to a rack and the caps are loosen to allow gas exchange.
10. Rack is placed in an incubator at 37ºC, 5% CO_2, 90% humidity.
11. Medium is changed every other day by aspirating off as much medium as possible without disrupting the pellet and replacing with 500µl of fresh complete chondrogenic medium.
12. After 21 days in culture the cell pellets are harvested by aspirating off all medium and washing twice in D-PBS.
13. Pellets are either fixed in formaldehyde or allowed to air dry and then either stained or stored at -20ºC until required for GAG measurement.

Adipogenic differentiation of hMSCs

I. MATERIALS AND EQUIPMENT

Solutions

1. Complete hMSCs medium
2. Fetal bovine serum (PAA, Austria, cat. No. A11-532 and A15-532, lot No. A53206-0618)
3. DMEM high-glucose (Sigma-Aldrich, USA)
4. Dexamethosone (Sigma-Aldrich, USA)
5. 3-Isobutyl-1-Methyl-Xanthine 500 mM (Sigma-Aldrich, USA)
6. Indomethacin (Sigma-Aldrich, USA)
7. Insulin (Sigma-Aldrich, USA)
8. Penecillin/Streptomycin (Invitrogen, USA)
9. 0.25% Trypsin-1mM EDTA
10. Dulbecco's phosphate buffered saline (D-PBS)
11. Flasks of hMSC ready for subculture
12. Isopropyl alcohol (IPA)

Materials

1. 6 well cell culture plates
2. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
3. Sterile filter pipette tips
4. Sterile 15 ml centrifuge tubes
5. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Timer
6. Haemocytometer with cover slip
7. Gilson pipettes (P10, P200 and P1000)
8. Pipette filler

II. PROCEDURE

1. Adipogenic induction media is prepared according to this table:

Reagent	Volume (to make 100 ml)	Final Concentration
DMEM high-glucose	87.6 ml	
Dexamethasone 1 mM	100 µl	1 µM
Insulin 1 mg/ml	1 ml	10 µg/ml
Indomethacin 100 mM	200 µl	200 µM
3-Isobutyl-1-Methyl-Xanthine	100 µl	500 µM
FBS	10 ml	10%
PNC/STM	1ml	100 U/ml PNC, 100 µg/ml STM

2. Adipogenic maintenance media is prepared according to this table:

Reagent	Volume (to make 100 ml)	Final Concentration
DMEM high-glucose	88 ml	
Insulin 1 mg/ml	1 ml	10 µg/ml
FBS	10 ml	10%
PNC/STM	1ml	100 U/ml PNC, 100 µg/ml STM

3. Cells are trypsinized, neutralised and counted.
4. $2x10^5$ MSCs are seeded / well.
5. 3 control wells and 3 adipogenic test wells are set up and marked.
6. Required number of cells for 6 wells is placed into a 15ml tube.

7. Cells are resuspended and diluted until the required number of cells are in 12ml media.
8. Cells are plated out 2 ml/well in a 6 well plate.
9. Plates are placed in an incubator at 37°C, 5% CO_2, 90% humidity.
10. After 24 hours cells should have adhered to the surface of the plate.
11. Cells are allowed to grow until they reach confluency. Medium is changed every 3 days.
12. Once confluent, 2 ml of the adipo induction media is added into the test wells and left for 3 days. Control wells receive normal growth medium.
13. After 3 days media in test wells is exchanged and 2 ml of maintenance media is added per test well and left for 1 day.
14. Steps 11-13 are repeated until 3 cycles in induction and maintenance media are completed.
15. For the final maintenance cells are left in the maintenance media for a total of 5-7 days.
16. Cells are then washed with D-PBS, formaldehyde fixed and Oil red O stained.

Results

Clinical application of MSCs will require expansion and differentiation of primary MSCs in order to obtain sufficient therapeutic cell numbers as it is not possible to get enough MSCs for clinical use during a single expansion. Usually, the cells have to be passaged 1-2 times in media that contains animal-derived reagents, which increases the risk of contamination of the cell culture and also increases the time from the bone marrow harvest to the final production of the cellular treatment to 4-6 weeks. Currently, there are commercially available serum-free media. Mesencult-ACF from Stem Cell Technologies and Thera Peak from Lonza were tested during the PurStem project. Unfortunately, the use of current serum-free conditions in culturing human MSCs selects for a subpopulation of cells that can survive serum deprivation and continue proliferating. A new complement of medium, growth factors, cytokines, etc. must therefore be developed specifically ensure consistent MSC cell surface receptor expression yielding uniform expansion of the MSC population in culture.

Comparison of PurStem media and commercial serum-free media

Colony forming assays and expansion cultures were set up in standardized PurStem (PS) media, which contains serum, low serum media (2% fetal bovine serum (FBS) and two commercially available serum-free media (Mesencult-ACF and Thera Peak). The cellular morphologies of the cultures were compared after 14 and 30 days.

MSC colonies were successfully cultured in commercially available serum-free media (Mesencult-ACF, Thera Peak) over the short term (14 days). After 14 days, the colonies observed in the standardized PurStem media were large, with densely packed cells, while those from the Mesencult-ACF media were small, containing fewer and more loosely packed cells. After 30 days, the cells in serum free media failed to proliferate and morphological changes were observed while the cells in PurStem media were confluent. In Therapeak media, spheroids were visible following the first passage. Despite the same initial seed concentration, the Mesencult-ACF cultures contained only 25% of the number of cells of the PurStem media cultures (Figure 3). Although our results demonstrated that it is possible to culture MSCs in commercially available serum-free media, these media were not suitable for sustaining MSCs in long term culture. Therefore, improved serum-free media conditions are required to culture MSCs for use in clinical therapy.

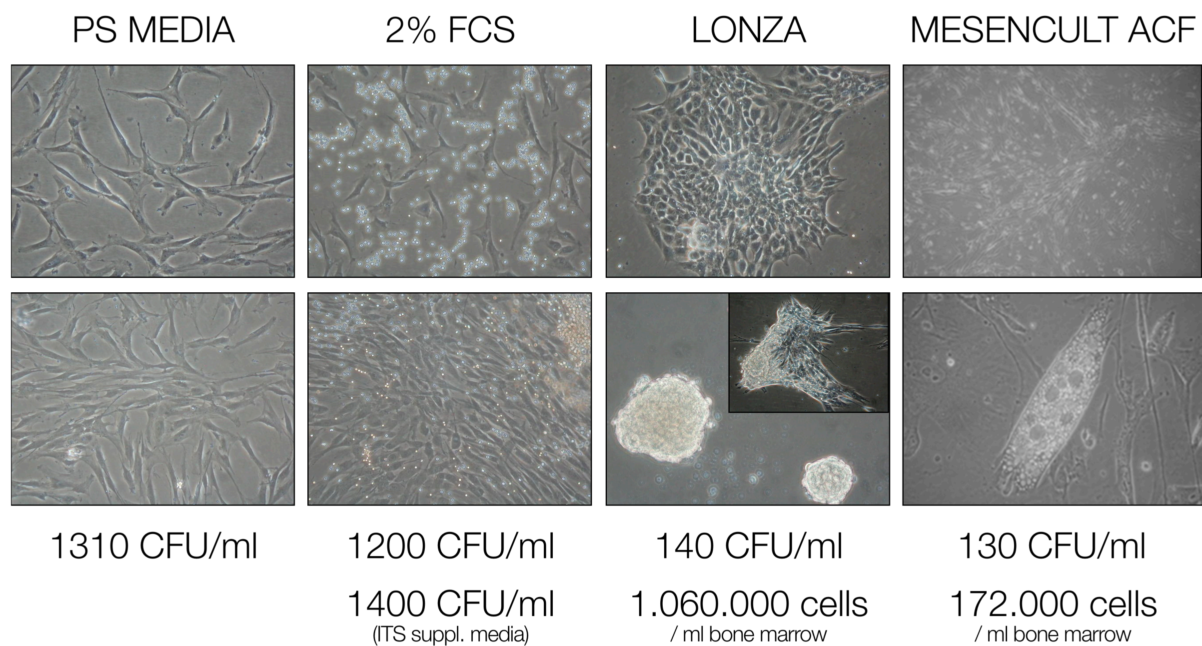


Figure 3. MSCs after 30 days in culture in PS (serum-containing), low serum (2% FCS), Lonza Thera Peak (serum-free) and Mesencult-ACF (serum-free) media.

Effect of Growth Factor Combinations and ECM Molecules on MSC Viability and Proliferation

In the beginning of the project PurStem team leaders compiled a list of relevant factors with the potential enhance MSCs cell attachment and proliferation, including mitogenic factors and ECM molecules, for screening (Table 6). A serum-free media from the literature developed for human articular chondrocytes, containing a cocktail of recombinant growth factors to drive cells towards osteogenic or chondrogenic differentiation was also tested. MSCs cells cultured under these serum-free conditions were elongated and fibroblast-like, similar to those cultured in PS media, and retained the ability to undergo osteogenic differentiation.

Cell Surface Antigens	Signalling Pathway
CD9, CD273, CD340, EGFR	EGF Receptors
CD126, CD130, CD132, CD210	IL Receptors
CD118, CD119, CD221	Interferon Family Receptor
CD91, CD105, CD273, TGFBR2	TGF-beta Superfamily Receptors
MET	HGF Receptor
Leukotriene B4 Receptor	Leukotriene Receptor

Cell Surface Antigens	Signalling Pathway
CD140a, CD104b	PDGF Receptors
CD183, CDw198, fMLP receptor	Chemokine Receptors
CD95, CD137 ligand, Lymphotoxin Receptor, TWEAK	TNF Receptors

Table 6. Growth factor receptors expressed in MSCs. Included here is a complete list of growth factor receptors expressed either at a MFI over 10 or in greater than 10% of the cell population.

MSCs attachment after 24 h was examined using a range of ECM molecules, including vitronectin, fibronectin, laminin and hyaluronic acid. For MSCs isolated in the presence of serum, vitronectin seemed to result in the best initial cell attachment without altering cell morphology (Figure 4). However, after 6 days, MSC proliferation was greatest on fibronectin coated plates. Further study showed that fibronectin promotes MSCs attachment by phosphorylation of protein FAK after 90 minutes.

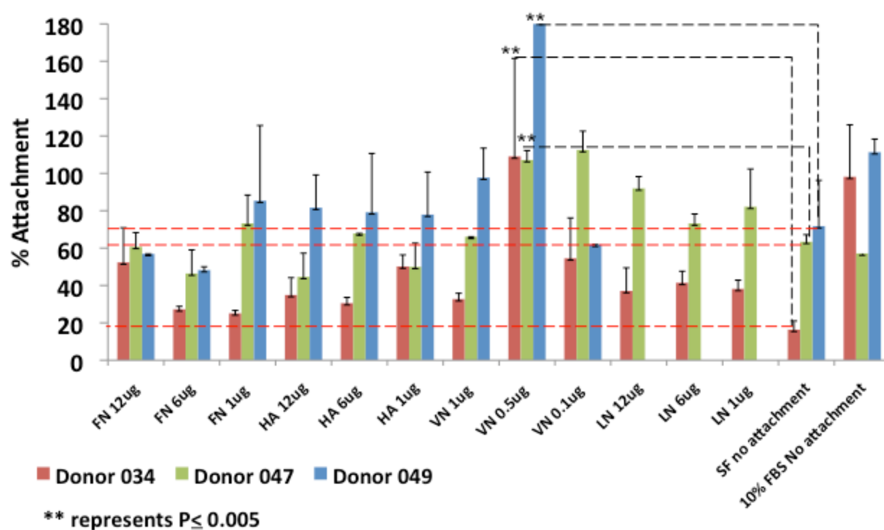
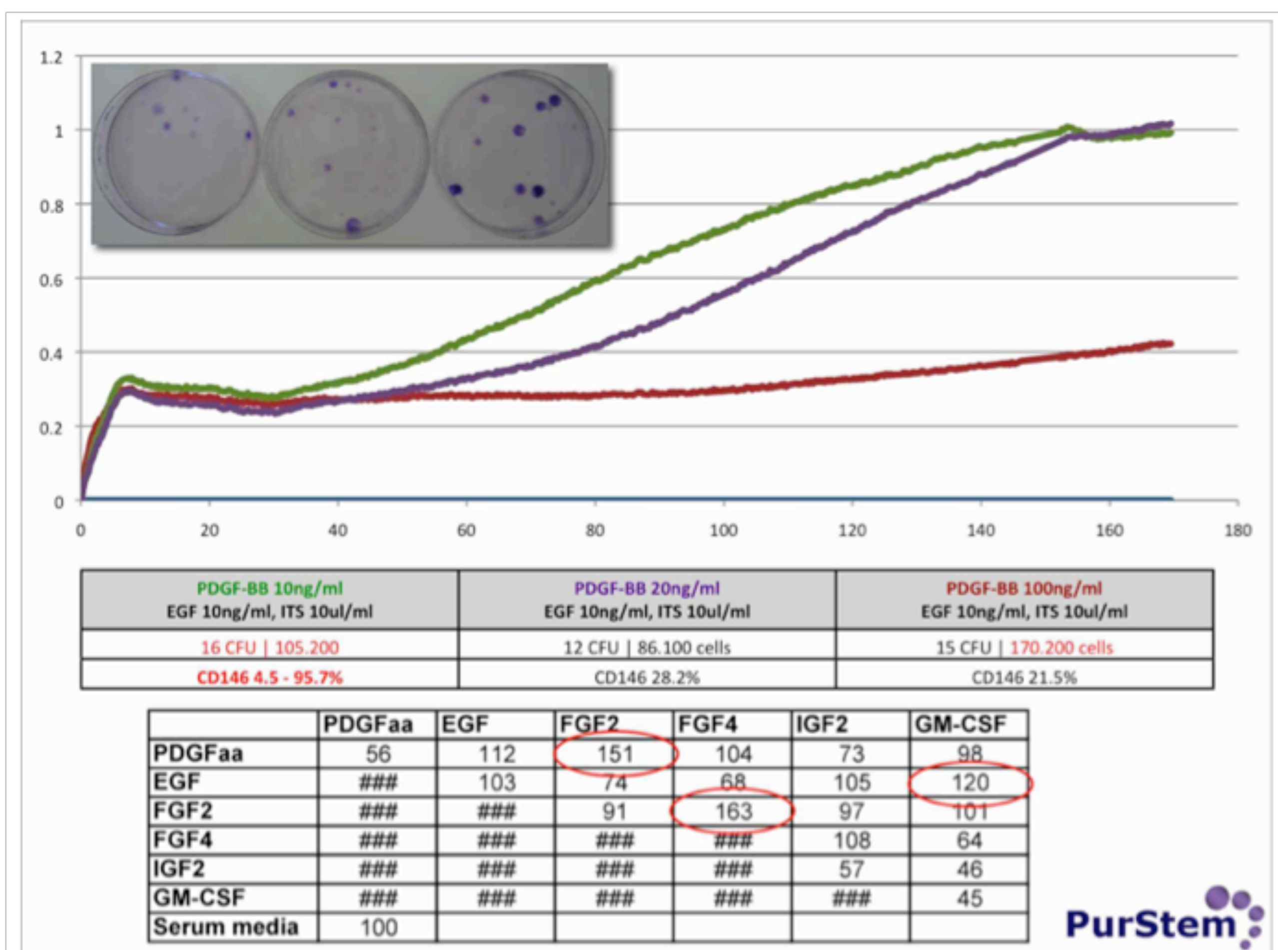


Figure 4. MSC attachment studies for MSC seeded on tissue culture plastic coated with various ECM molecules including fibronectin (FN), hyluronic acid (HA), vitronectin (VN) and laminin (LN) cultured in serum free media. MSC seeded on tissue culture plastic in serum free media with no coating were used as controls.

MSCs proliferation in the presence of growth factors on fibronectin coated plates in serum free media was examined. The growth factors examined included PDGF-AA, bFGF, FGF4, IGF2, EGF and GM-CSF. Initial results suggested that bFGF and FGF4 play a role in proliferation. Further optimization of the serum-free media formulation was conducted, and western blot analysis revealed that the presence of EGF in serum-free media promoted MSC attachment, but did not

promote cell proliferation. There was a statistically significant increase in cell proliferation for media with PDGF-AA/FGF2 or FGF4/FGF2 or FGF4 compared to serum free media. There was no statistically significant difference between MSCs cultured in FGF2/FGF4 and FGF4 alone.

Using colony forming assays and xCELLigence technology which allows monitoring of cellular events in real time, several other combinations of growth factors/supplements were tested and high throughput analysis of proliferation and viability was performed (Figure 5). Basic combinations of growth factors/supplements with serum-free media were tested using high throughput analysis of proliferation, viability assessment and phenotyping. Cells cultivated in high concentrations of PDGF-BB were more numerous but less stable during long term cultivation. Moreover CD146 expression decreased as the amount of PDGF-BB used was increased. A combination of EGF (10 ng/ml) and ITS (10 µl/ml) with the addition of HSA (Human serum albumin) (0.125%) yielded an optimum basal serum-free media composition.



PDGF-BB 10ng/ml EGF 10ng/ml, ITS 10ul/ml	PDGF-BB 20ng/ml EGF 10ng/ml, ITS 10ul/ml	PDGF-BB 100ng/ml EGF 10ng/ml, ITS 10ul/ml
16 CFU | 105.200	12 CFU | 86.100 cells	15 CFU | 170.200 cells
CD146 4.5 - 95.7%	CD146 28.2%	CD146 21.5%

	PDGFaa	EGF	FGF2	FGF4	IGF2	GM-CSF
PDGFaa	56	112	151	104	73	98
EGF	###	103	74	68	105	120
FGF2	###	###	91	163	97	101
FGF4	###	###	###	###	108	64
IGF2	###	###	###	###	57	46
GM-CSF	###	###	###	###	###	45
Serum media	100					

Figure 5. MSC proliferation in serum free media containing various growth factors. Values represent % proliferation compared to MSC cultured in the presence of serum (n=2). Comparison of xCELLigence patterns for 3 different media is also shown (PDGF-BB 10 ng/ml, 20 ng/ml and 100 ng/ml).

Surface markers of MSCs cultured in serum-free conditions

To assess the effect of the supplements, CD146 and CD271 expression levels on the cells were examined at frequent time points. Cells grown under standardized PurStem conditions (10% serum) had a standard MSC phenotype (Figure 7). Cells grown in other media conditions (Figure 6) had an MSC phenotype (expressing CD73, CD90, etc. and lacking expression of lineage markers) but did differ in the expression levels of some markers. The most prominent difference was a decrease in expression of CD105 and CD146 for the three media tested and an increased expression of CD271 for Medium A when compared to cells grown in standardized Purstem media (Figure 8).

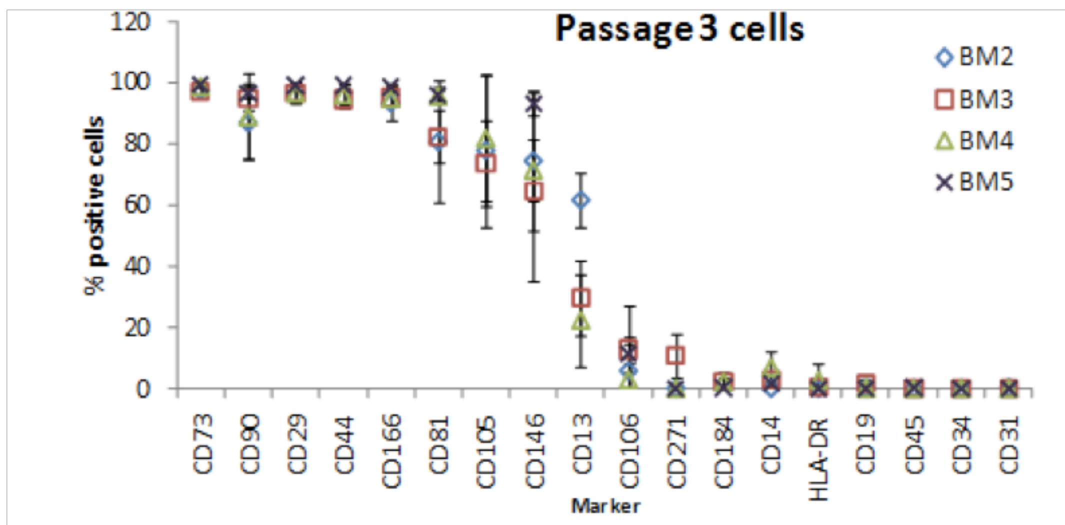
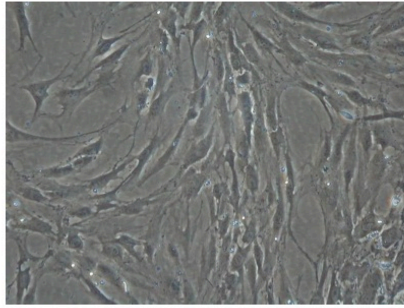
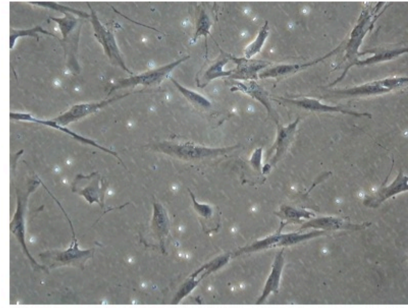
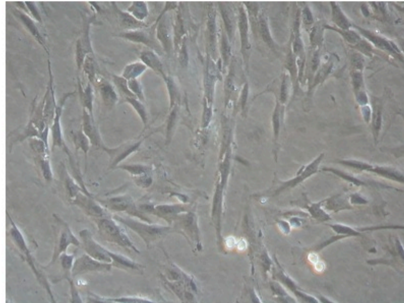


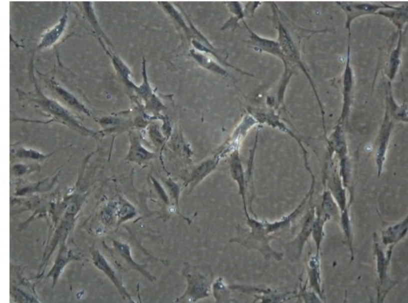
Figure 7. Phenotype of cultured MSCs (passage 3) from all donors cultured under standardized PurStem conditions in the project partner laboratories. Data points show the average value for each donor across the 4 partner laboratories. Error bars show the standard deviation of the values between different centers.

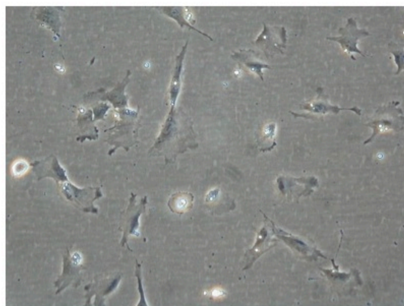
MSCs were also cultivated in special culture dishes (PureCoat Amine and Carboxyl, BD) to determine if cells could be cultured in serum-free media, supplemented with EGF, without plate coating to meet GMP requirements. PureCoat Amine dishes were suitable for serum-free culture, while PureCoat Carboxyl was unsuitable (Figure 9).

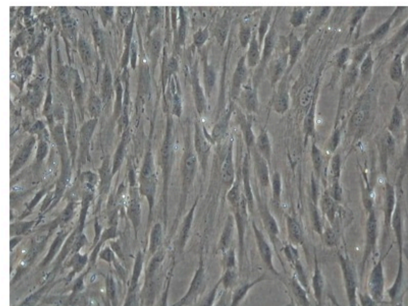

Medium A

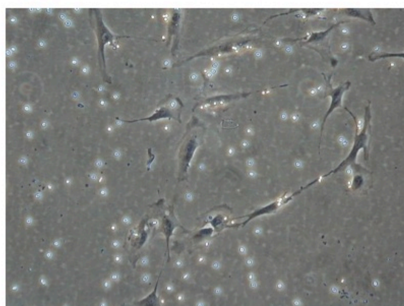

Medium A + FGF7


Medium A + FGF4


Medium A + GM-CSF


Medium A + FGF4 + GM-CSF


Medium A + Activin A


Medium A + IGF-1

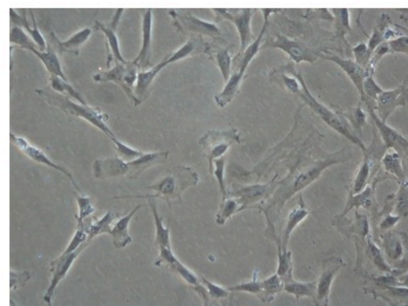

PS medium

Figure 6. Medium A vs PS medium – comparison of morphology in primary culture at day 7

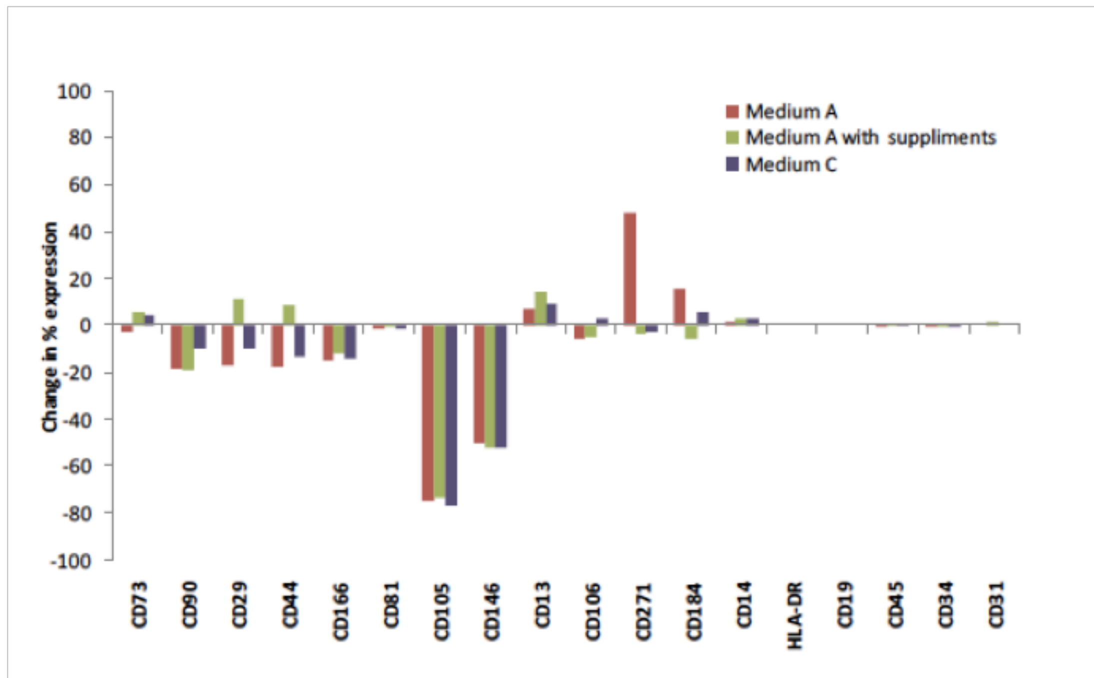


Figure 8. Difference in expression of cell surface markers of MSCs cultured in different media relative to MSC cultured under standardized PurStem conditions. Phenotypes of cells at Passage 2 were compared (Medium A (1% FCS, EGF, ITS), Medium A with supplements - IGF1 +FGF4 +Activin A and Medium C (Medium A with lower concentration of Ascorbic acid).

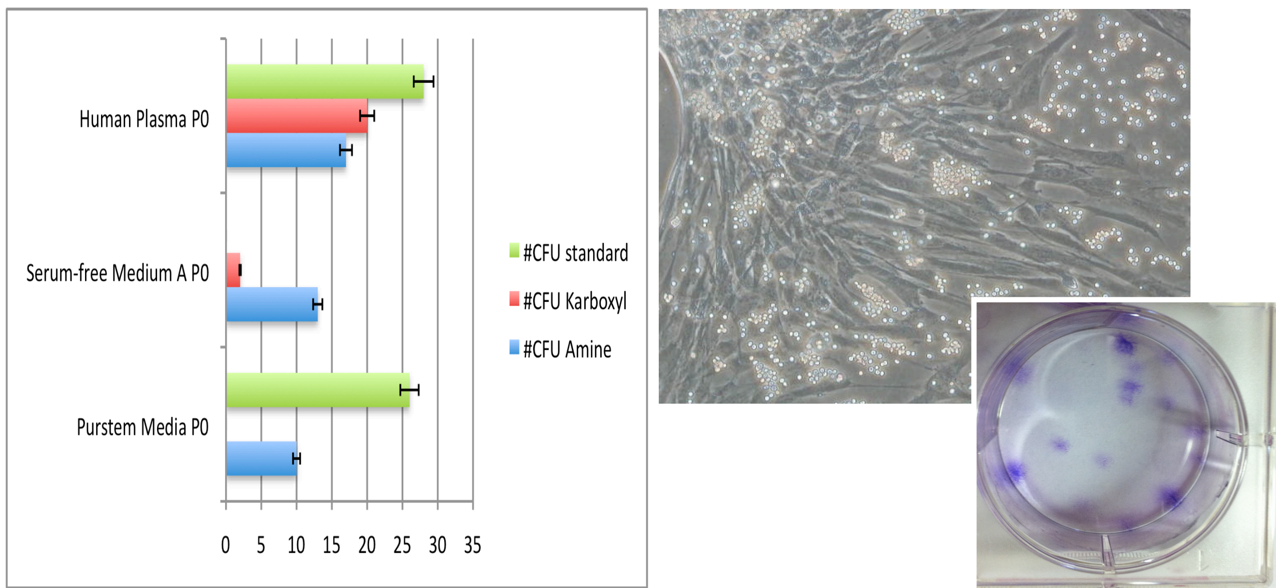


Figure 9. PureCoat Amine surface has been tested as beneficial for serum-free cultivation. No. of CFUs, crystal violet stained colonies (serum-free medium) and phase contrast microscopy of serum-free sample are presented. PureCoat Carboxyl surface is not suitable for both serum-free media and FCS containing media. Human plasma containing samples successfully proliferated in all culture vessels.

According to the phenotypic data (Figure 10), high throughput analysis of proliferation, viability assessment, and PEf, PureCoat Amine dishes were suitable for serum-free culture, while PureCoat Carboxyl were unsuitable.

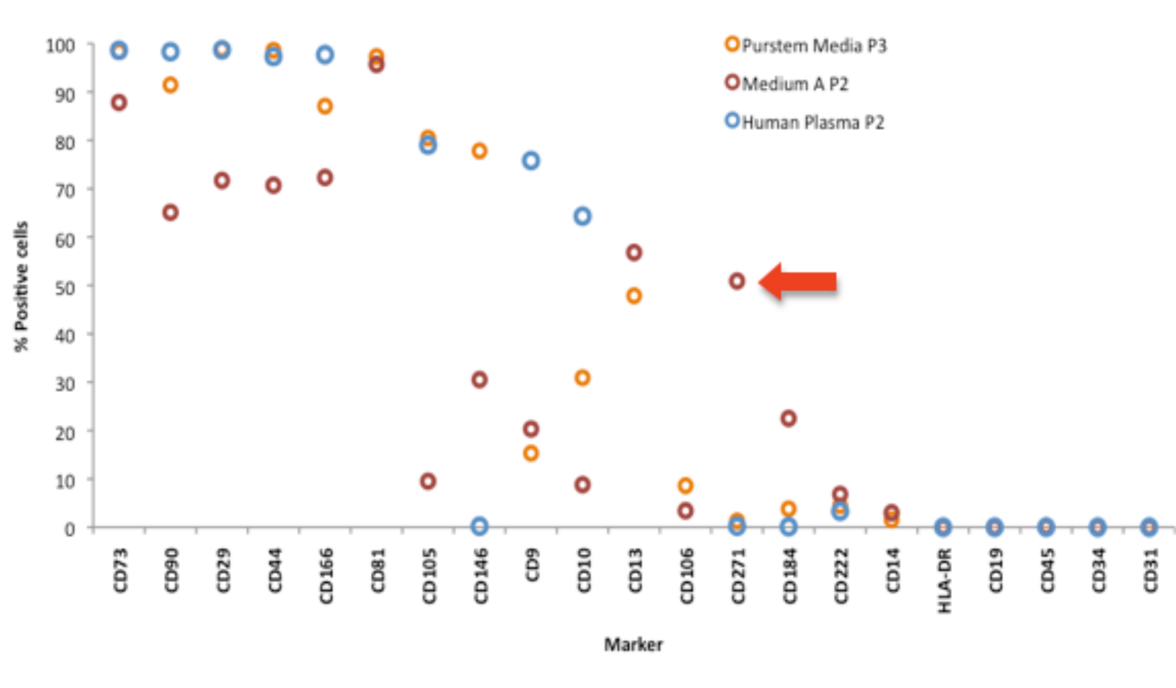


Figure 10. Phenotypic analysis of directly plated MSCs (P2, P3), three different media were tested (Medium A is the serum-free medium), red arrow is pointing moderate expression of CD271. Serum-free medium was the only one where CD271 positivity remained till P3.

Differentiation potential of MSCs cultured in serum-free conditions

Moreover, the potential whether the MSCs grown under the optimized PurStem serum-free conditions differentiated towards mesenchymal lineages was examined.

The serum-free medium contained a cocktail of recombinant growth factors specifically used to drive cells towards osteogenic or chondrogenic differentiation, in association with other components involved in normal cell homeostasis and metabolism. The osteo- and chondrogenic potential of serum free expanded cells was compared with the potential of cells grown in serum containing media (Figure 11, Figure 12). Results indicated that MSCs cultured under the PurStem serum-free conditions show an elongated and fibroblast-like phenotype. Nonetheless, they maintain the ability to undergo osteogenic and chondrogenic differentiation.

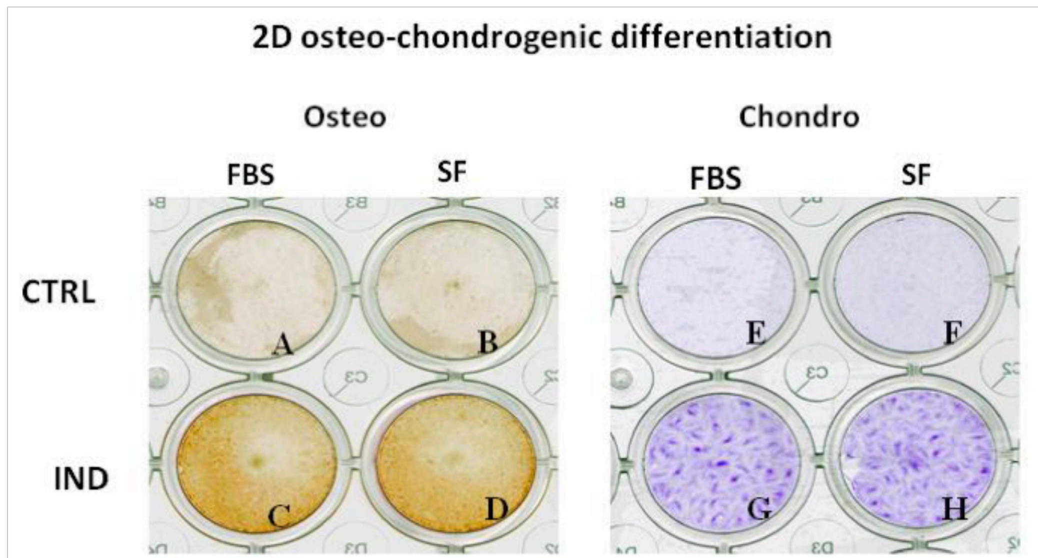


Figure 11. MSCs differentiation was induced with an osteogenic medium for 3 weeks in 10% FBS medium (C) or serum-free medium (D) or with a chondrogenic medium for 2 weeks in 10% FBS medium (G) or serum-free medium (H). Control cells were maintained in 10% FBS medium (A, E) or serum-free medium (B, F).

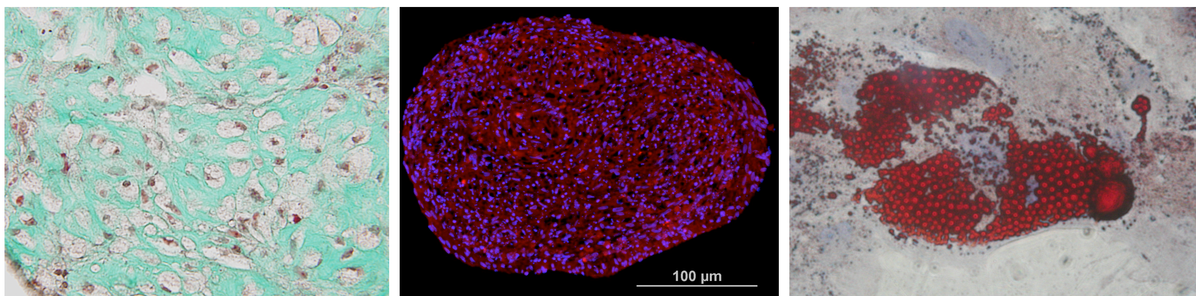


Figure 12. (from the left) Osteogenic 3D differentiation (3 weeks, green trichrome); Chondrogenic 3D differentiation (2 weeks, col II. detection Cy3, DAPI); Adipoogenic differentiation (2 weeks, oil red staining).

The differentiation ability of dental pulp derived MSCs cultured under serum free conditions was also determined. (Figure 13).

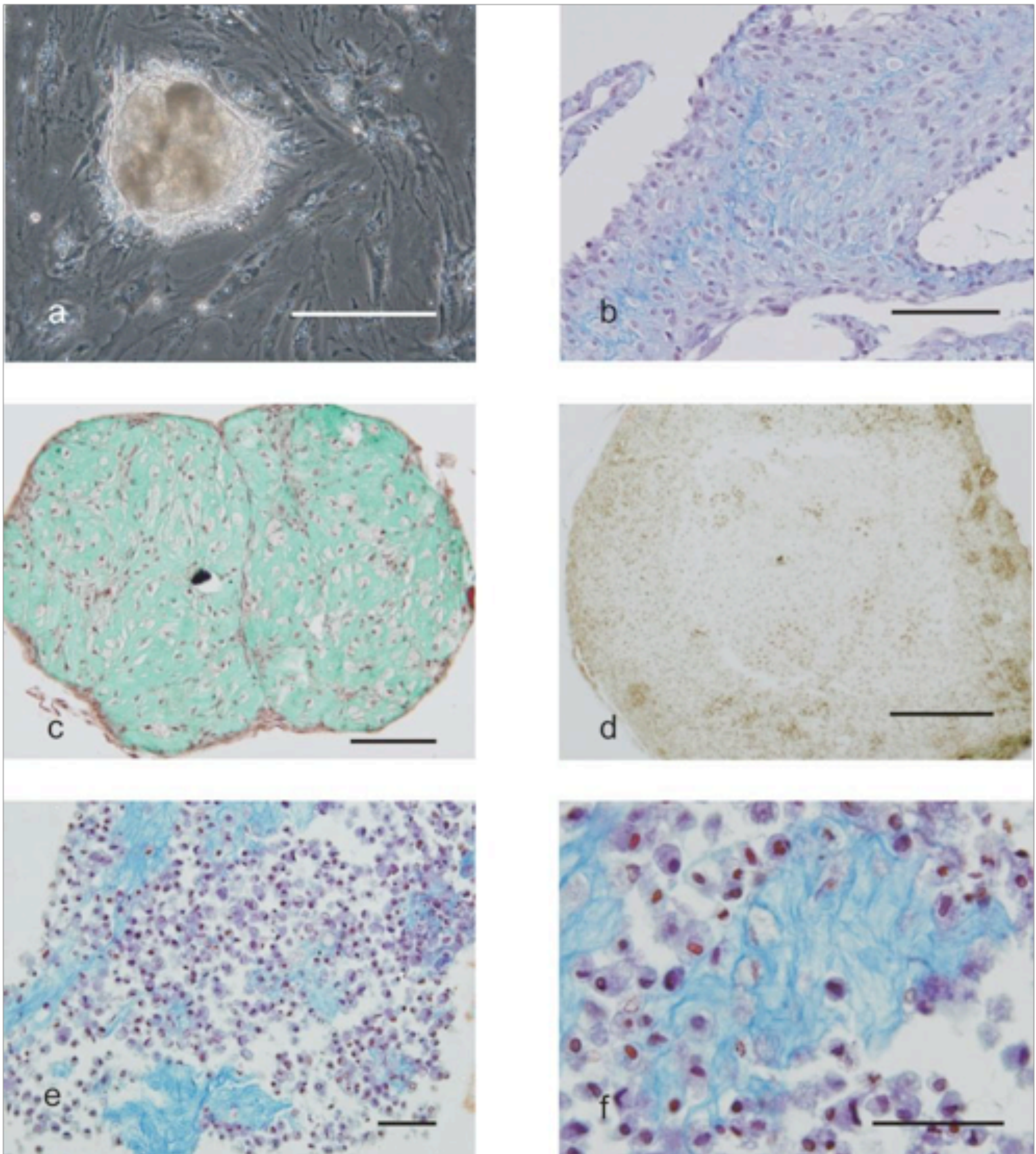


Figure 13. a-f Osteogenic and chondrogenic differentiation of DPSCs. a Phase contrast micrograph of osteogenic nodule after 4 week-cultivation in differentiation medium. b Osteoblast-like cells surrounded by extracellular matrix containing collagen fibres; Ladewig blue trichrome. c Histochemical staining confirmed a rich production of collagen in micro-mass pellets; Masson's green trichrome. d Immunoperoxidase histochemistry of osteonectin confirmed the presence of this bone-specific glycoprotein that links minerals to collagen within the micro-mass pellets. e-f Chondrogenic micro-mass bodies were composed of cells and extracellular matrix. Alcian blue staining confirmed the presence of sulphated acidic glycosaminoglycans, a principal component of cartilaginous ground substance. In a detailed view (f), oval morphology of chondroblast-like cells entrapped in the matrix was well visible. Scale bars a-c 100 .m, d 200 .m, e-f 50 .m.

Serum-free cryopreservation

In classic serum-containing freezing media, serum exerts a protective function for the cells. BIOFREEZE (Biochrom AG, Germany) is a commercially available media free of animal-derived substances that is also free of genetically modified organisms. MSCs (passage 1) cells cultivated in serum-free media supplemented with EGF were cryopreserved using the standardised PurStem cryopreservation procedure and BIOFREEZE for comparison. There was no significant difference between the number of cells frozen and number of thawed viable cells (Figure 14). The number of recovered cells at passage 2 and viability of the samples immediately after thawing was significantly better for the standardised PurStem procedure than the BIOFREEZE procedure. However, after one consecutive passage (Passage 2), the viability of both protocols was comparable.

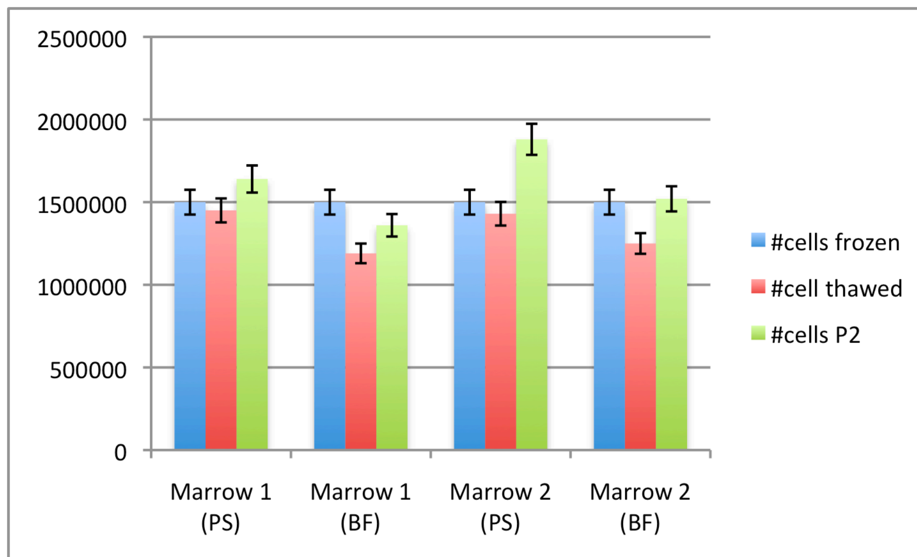


Figure 14. Cell No. after thawing and at P2. Standard PurStem (PS) cryopreservation protocol vs BIOFREEZE serum free cryopreservation media (BF).

PurStem serum-free media

As described previously, the key outputs of this thesis are standardized procedures. Subsequent protocols serve as uniform methods of MSC serum-free isolation, culture and cryostorage.

Standard operating protocols are separated into five sections:

A) MSCs Serum Free Isolation Protocol
B) MSCs Serum Free Sub Culture Protocol
C) MSCs Serum Free Cryopreservation Protocol
D) MSCs Serum Free Differentiation Assays
E) xCELLigence MSCs profiling

A) MSCs Serum Free Isolation Protocol

I. MATERIALS AND EQUIPMENT

Solutions

1. Human bone marrow sample
2. Complete hMSC serum free medium
3. Dulbecco's phosphate buffered saline (D-PBS)
4. 4.0% (v/v) acetic acid
5. 70% isopropyl alcohol (IPA) or industrial methylated spirits (IMS)
6. 0.25% trypsin-1mM ethylenediaminetetraacetic acid (EDTA)

Materials

1. Sterile serological pipettes (25ml, 10ml, 5ml)
2. Sterile pipette tips (P100 & P1000)
3. 1.5 ml snap-top microfuge tubes
4. 50 ml centrifuge tubes
5. BD PureCoat™ Amine 175 cm^2 Flask (cat. No. 356728), with vented cap
6. Disposable container for liquid waste

Equipment

1. Biological safety cabinet class II (BSC)
2. Incubator, (37^oC, 5% CO_2, 90% humidity)
3. Centrifuge
4. Haemocytometer with cover slip
5. Z2 Counter, ViCell analyser
6. Inverted light microscope
7. 4^oC refrigerator
8. Gilson pipettes (P100 & P1000)
9. Pipette filter
10. Calculator

II. SOLUTIONS

1. Basal medium is warmed to 37 ºC using a water bath
2. Basal medium is alpha MEM with Glutamax (Invitrogen, USA, cat. No. 32561-029)
3. Add 100 µM Ascorbic acid (cat. No. A8960, Sigma-Aldrich, USA)
4. Add 0.01 µM Dexamethasone (cat. No. D2915, Sigma-Aldrich, USA)
5. Add 100 units/ml of penicillin and streptomycin (cat. No. 15140, Invitrogen, USA)
6. Store media at 4ºC until use
7. Add 10 ng/ml AF-EGF (cat. No. AF-100-15, PeproTech, USA) and 10µl/ml ITS supplement (cat. No. I3146, Sigma-Aldrich, USA) immediately before using media.
8. For enhancing the yield with the loss of CD271 expression, Activin A (cat. No. 120-14, PeproTech, USA) can be added immediately before using media in concentration 10 ng/ml

III. COLLECTION OF HUMAN BONE MARROW

Human bone marrow is either aspirated or purchased, then aliquoted and sent to the laboratory. Sodium heparin is used as the anti coagulant for the aspiration.

Donors are treated in accordance with the local donor programme and in full compliance with ethical approval.

IV. hMSCs ISOLATION PROCEDURE

hMSCs are isolated by direct plating (i.e. plastic adherence plastic adherence to BD PureCoat™ Amine surface).

Isolation Day 0

1. The interior work area of the BSC is wiped with alcohol.
2. D-PBS and complete hMSC serum free media are transfered to the BSC.
3. Human bone marrow sample is transfered to the interior workspace of the clean and operating BSC.
4. Human bone marrow aspirate is carefully transfered from the transportation tubes into a sterile 50 ml centrifuge tube. Total volume of the sample is measured with a sterile serological pipette.
5. Total bone marrow aspirate volume is aliquoted into several 50 ml centrifuge tubes and diluted 1:1 with D-PBS for the initial wash. The suspension is mixed by inverting the tube several times gently.
6. Tubes are centrifuged at 900 g (2000rpm) for 10 minutes at room temperature.
7. Tubes are carefully removed from the centrifuge and carefully placed back into the BSC as the cell pellets are very soft and can be easily disturbed.

8. Using a sterile serological 25 ml pipette, as much of the supernatant as possible is carefully removed without disturbing the cell pellets.

9. hMSCs are resuspended again in 40 ml of D-PBS.

10. Pellets are then combined in a fresh sterile 50 ml tube. If multiple tubes have been used, the tubes are rinsed with a total volume of 5 ml D-PBS and this wash is added to the combined pellets. Total volume of cell suspension is measured with a sterile serological pipette.

11. 50 µl aliquot of the bone marrow suspension is removed for cell counting using a P100 pipette, and added to 450 µl of D-PBS in a microfuge tube. Suspension is then mixed by closing the lid tightly and flicking with finger once or several times until mixed well.

12. 50 µl of 4% (v/v) acetic acid is pipetted into a microfuge tube.

13. From the bone marrow/D-PBS suspension, a 50 µl aliquot is removed and added to the 4% (v/v) acetic acid in the microfuge tube. Suspension is mixed and let sit for at least one minute so that the erythrocytes have sufficient time to lyse from exposure to the acetic acid.

14. Suspension is pipetted onto a haemacytometer. Number of unlysed cells is counted. The cell number/ml is calculated by multiplying the number of cells counted in the 25 square grid by the dilution factor of 20 and then multiplying by 10^4. The total cell number is counted by multiplying the cell number/ml by the total suspension volume.

15. Cell number and viability are then verified by Z2 Counter (Beckman Coulter, USA) and ViCell analyser (Beckman Coulter, USA).

16. Primary hMSC are plated at a cell density 25,000 cells/cm^2. The number of T-175 flasks required for the number of cells counted is set. The volume of complete hMSC medium necessary to be added to the cell suspension is counted, so that there is enough volume to allow 5ml of the cell suspension to be pipetted into each flask. This volume is added to the cell suspension.

17. The volume of complete hMSC serum free medium calculated earlier is added to the cell suspension.

18. 25 ml of complete hMSC serum free medium supplemented with EGF and ITS is added into each T-175 flask evenly such that the cell suspension is evenly distributed in the flask. Volumes are adjusted appropriately when using different sized flasks.

19. 5 ml of the cell suspension is added into each flask. The cell suspension have to be spread evenly through the flask.
20. The flasks of cells lying horizontally are placed in an incubator at 37^oC, 5% CO_2, 90% humidity.

Day 4

1. Interior workspace of the BSC is wiped down with alcohol.
2. All solutions are transfered to the BSC.
3. Cell culture flasks are removed from the incubator.
4. The outside of the flasks is wipe down with alcohol and transfered to the BSC.
5. Flasks are gently swirled to dislodge the red blood cells and other unattached cells.
6. Media containing the unattached cells is removed using a sterile serological pipette.
7. 10ml D-PBS is added to each flask, flasks are swirled to suspend the unattached cells, and D-PBS is aspirated without touching the monolayer.
8. 35ml of warm, fresh complete hMSC serum free medium supplemented with EGF and ITS is added.
9. Individual attached cells are observed under the inverted light microscope at 4x magnification.
10. Flasks are finally returned to the incubator.

Subsequent feeding of cultured hMSCs

1. Note: hMSCs cultures are to be fed twice a week.
2. Interior workspace of the BSC is wiped down with alcohol.
3. All solutions are transfered to the BSC.
4. Cell culture flasks are removed from the incubator.
5. The outside of the flasks is wipe down with alcohol and transfered to the BSC.
6. Conditioned medium is removed from the flask by aspiration with a sterile aspiration pipette.
7. 35ml of warm, fresh complete hMSC serum free medium supplemented with EGF and ITS is added.
8. Attached cells are observed under the inverted light microscope at 4x magnification.
9. Flasks are returned to the incubator.

B) MSCs Serum Free Sub Culture Protocol

I. MATERIALS AND EQUIPMENT

Solutions

1. 0.25% Trypsin-1mM EDTA
2. Complete hMSC serum free medium
3. Dulbecco's phosphate buffered saline (D-PBS)
4. Flasks of hMSC ready for subculture
5. Isopropyl alcohol (IPA)

Materials

1. BD PureCoat™ Amine 175 cm^2 Flask (cat. No. 356728), with vented cap
2. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
3. Sterile filter pipette tips (for P100)
4. Sterile 15 ml centrifuge tubes
5. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4^oC refrigerator
3. Centrifuge
4. Incubator (37^oC, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Calculator and Timer
7. Haemocytometer with cover slip
8. Z2 Counter, ViCell analyser
9. Gilson pipette (P100)
10. Pipette filler

II. PROCEDURE

Note: The primary MSCs culture should be passaged when discrete colonies of MSCs have formed, but before multilayer growth occurs. In subsequent passages, MSCs should be subcultured at 80-90% confluence.

1. A few hours before beginning the procedure, 0.25% Trypsin-1mM EDTA is removed from the freezer, complete hMSC serum free medium from the 4^oC refrigerator and D-PBS from room temperature storage and are allowed to come to room temperature.
2. The BSC is turned on and the interior work area is wiped down with alcohol.
3. Wiped containers with solutions at ambient temperature for a minimum of one and a half hours are placed in BSC.

4. Flasks with hMSC are removed from the incubator, wiped down with alcohol and placed in the BSC.
5. Medium from each flask is aspirated using a 25 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
6. 10 ml D-PBS is added to each T-175 flask using a 10 ml sterile serological pipette to wash the hMSC. (volumes are adjusted according to the volume of culture flasks)
7. D-PBS is aspirated using a 10 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
8. 5 ml 0.25% Trypsin – 1mM EDTA (alternatively Accutase or Accumax) is added to each flask using a 5ml sterile serological pipette. (volumes are adjusted according to the volume of culture flasks)
9. Flasks are incubated at room temperature for 5-8 minutes.
10. Cells are observed using an inverted light microscope to ensure that the hMSC have dislodged from the surface of the flask. If they are still adherent, flasks are incubated at room temperature until the hMSC are in suspension. Flasks are tapped sharply with gloved hand to ensure detachment.
11. The length of incubation time of hMSC in 0.25% Trypsin-1mM EDTA is recorded.
12. 0.25% Trypsin – 1mM EDTA is inactivated by adding 10 ml of prepared complete hMSC serum free medium without EGF and without ITS to each flask using a sterile serological pipette.
13. The suspension is pipetted over the bottom surface of the vessel several times to fully dislodge the hMSC, and then the cell suspension is transfered to a sterile 15 ml centrifuge tube. Suspension is centrifuged for 10 minutes at 900 g.
14. The interior work area of the BSC is wiped down with alcohol.
15. Centrifuge tubes are transfered from the centrifuge to the BSC. The supernatant is aspirated off and discarded in the container of bleach.
16. Cell pellets are resuspended and pooled from each centrifuge tube in a known volume of complete hMSC serum free medium supplemented with EGF and ITS (5-10 ml). The total volume of the final hMSC suspension is measured.
17. 50 µl aliquot of the hMSC cell suspension is removed for cell counting.
18. Cell number/ml is calculated by multiplying the number of cells counted in the 25 square grid by 10^4. Total cell number is calculated by multiplying the cell number/ml by the total volume of the cell suspension.
19. 4,500 cells/cm^2 are plated in 35 ml of prepared complete hMSC serum free medium supplemented with EGF and ITS in each T-175 flask. The flasks of cells lying

horizontally are placed in an incubator at 37°C, 5% CO_2, 90% humidity. (Note: Ensure the cells are evenly distributed in the flask by gently rocking.)

20. hMSC are fed twice per week and observed under an inverted light microscope before they are fed.
21. Note: When hMSC have formed a semi-confluent monolayer, decide whether to subculture the hMSC again, or cryopreserve.

C) MSCs Serum Free Cryopreservation Protocol

I. MATERIALS AND EQUIPMENT

Solutions

1. Flasks of hMSC ready for cryopreservation
2. Complete hMSC serum free medium
3. 0.25% trypsin – 1mM EDTA
4. Biofreeze cryopreservation medium (cat. No. F2270, Biochrom AG, Germany)
5. Isopropyl alcohol (IPA)

Materials

1. Serological pipettes (25 ml, 10 ml, 5 ml)
2. Sterile pipette tips (for P100)
3. Sterile 50ml tube
4. Sterile 15 ml centrifuge tubes
5. Sterile 1ml cryovials
6. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Timer
7. Haemocytometer with cover slip
8. Z2 Counter, ViCell analyser
9. Mr Frosty
10. -80°C freezer
11. Gilson pipettes (P100 & P1000)
12. Pipette filler
13. Liquid nitrogen freezer

II. PROCEDURE

1. A few hours before beginning the procedure, 0.25% Trypsin-1mM EDTA is removed from the freezer, complete hMSC medium from the 4°C refrigerator and D-PBS from room temperature storage and are allowed to come to room temperature.
2. The BSC is turned on and the interior work area is wiped down with alcohol.
3. Wiped containers with solutions at ambient temperature for a minimum of one and a half hours are placed in BSC.
4. Flasks with hMSC are removed from the incubator, wiped down with alcohol and placed in the BSC.
5. Medium from each flask is aspirated using a 25 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
6. 10 ml D-PBS is added to each T-175 flask using a 10 ml sterile serological pipette to wash the hMSC. (volumes are adjusted according to the volume of culture flasks)
7. D-PBS is aspirated using a 10 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
8. 5 ml 0.25% Trypsin – 1mM EDTA is added to each flask using a 5ml sterile serological pipette. (volumes are adjusted according to the volume of culture flasks)
9. Flasks are incubated at room temperature for 5-8 minutes.
10. Cells are observed using an inverted light microscope to ensure that the hMSC have dislodged from the surface of the flask. If they are still adherent, flasks are incubated at room temperature until the hMSC are in suspension. Flasks are tapped sharply with gloved hand to ensure detachment.
11. The length of incubation time of hMSC in 0.25% Trypsin-1mM EDTA is recorded.
12. 0.25% Trypsin – 1mM EDTA is inactivated by adding 10 ml of prepared complete hMSC serum free medium without EGF and without ITS to each flask using a sterile serological pipette.
13. The suspension is pipetted over the bottom surface of the vessel several times to fully dislodge the hMSC, and then the cell suspension is transfered to a sterile 15 ml centrifuge tube. Suspension is centrifuged for 10 minutes at 900 g.
14. The interior work area of the BSC is wiped down with alcohol.
15. Centrifuge tubes are transfered from the centrifuge to the BSC. The supernatant is aspirated off and discarded in the container of bleach.

16. Cell pellets are resuspended and pooled from each centrifuge tube in a known volume of complete hMSC medium without EGF and ITS supplement (5-10 ml). The total volume of the final hMSC suspension is measured.
17. 50 µl aliquot of the hMSC cell suspension is removed for cell counting.
18. Cell number/ml is calculated by multiplying the number of cells counted in the 25 square grid by 10^4. Total cell number is calculated by multiplying the cell number/ml by the total volume of the cell suspension.
19. Cell suspension is centrifuged for 10 minutes at 900 g at room temperature.
20. Centrifuge tubes are transfered from the centrifuge to the BSC. The supernatant is aspirated off and discarded in the container of bleach.
21. Cell pellet is resuspended in an appropriate volume of 4°C cold Biofreeze medium resulting in $1x10^6$ cells/ml ***(1 part pellet + 9 parts Biofreeze)***
22. The cell suspension is aliqoted into appropriately labeled cryovials.
23. Note: Ensure cryovials are labeled with the following - cell batch No., No. of cells/ vial, passage No., date, operators initials or name
24. Cryovials are placed into Mr. Frosty.
25. Mr Frosty is transfered to -80°C freezer and left there overnight.
26. After cryovials have been at -80°C freezer overnight, they are transfered to liquid nitrogen storage container.
27. Note: Record rack, box and position within box that the cryovials are placed.

D) MSCs Serum Free Differentiation Assays

Note: Cells for the MSC Serum Free Differentiation Assays are trypsinized and counted acc. to SOP B. (MSC Serum Free Sub Culture Protocol). Cell suspension is the subjected to the induction media – either in monolayer (osteogenic dif., adipogenic dif.) or in 3D cell pellet (chondrogenic dif.).

Osteogenic differentiation

I. MATERIALS AND EQUIPMENT

Solutions

1. 0.25% Trypsin-1mM EDTA
2. Complete hMSC serum free medium
3. Dulbecco's phosphate buffered saline (D-PBS)
4. Flasks of hMSC ready for subculture

5. Isopropyl alcohol (IPA)
6. serum-free osteogenic media - supplemented McCoy's medium containing 1% ITSb (BD Bioscience, USA), ascorbic acid (50 µM), β-glycerol phosphate (10 mM), and dexamethasone (100 nM) supplemented with BMP-2, BMP-4 and HGF (all PeproTech, USA).

Materials

1. BD PureCoat™ Amine 6-well culture plates
2. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
3. Sterile filter pipette tips (for P100)
4. Sterile 15 ml centrifuge tubes
5. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Calculator and Timer
7. Haemocytometer with cover slip
8. Z2 Counter, ViCell analyser
9. Gilson pipette (P100)
10. Pipette filler

II. PROCEDURE

1. According to previous SOP, cells are trypsinised, neutralised and counted. Then $3x10^4$ cells are seeded per well. 3 control wells and 3 osteogenic test wells are set up.
2. Required number of cells for 6 wells is placed into a 15 ml tube.
3. Cells are diluted in complete hMSC serum free medium until the final volume is 12 ml
4. 2 ml of cell suspension are plated out per well in a 6 well plate
5. Plates are placed in an incubator at 37°C, 5% CO_2, 90% humidity.
6. After 24 hours cells should have adhered to the plastic of the plate.
7. The medium is changed using 2 ml of complete hMSC serum free medium on control wells and 2 ml osteogenic medium on test wells.
8. Media is changed twice a week, ensuring the correct medium is added to each well.
9. Assay is harvested between 10 and 17 days depending on the condition of the monolayer. Cells need to be harvested before they peel as they will be stained.
10. 1 control and 1 test well are stained for Fast Violet and Von Kossa staining. The remaining 2 wells for each are utilised for calcium quantification.

Chondrogenic differentiation

I. MATERIALS AND EQUIPMENT

Solutions

1. 0.25% Trypsin-1mM EDTA
2. Complete hMSC serum free medium
3. Dulbecco's phosphate buffered saline (D-PBS)
4. Flasks of hMSC ready for subculture
5. Isopropyl alcohol (IPA)
6. serum-free chondrogenic media - high glucose DMEM supplemented with ITS + supplement, 100 nM dexamethasone, 1 mM sodium pyruvate, 50 µg/ml ascorbic acid-2-phosphate, Penicillin/Streptomycin, (all Sigma-Aldrich, USA), 40 µg/ml L-proline, TGFβ-3 20µg/ml (both PeproTech, USA).

Materials

1. Sterile siliconized pipette tips
2. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
3. Sterile filter pipette tips (for P10, P100, P1000)
4. Sterile 15 ml centrifuge tubes
5. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Calculator and Timer
7. Haemocytometer with cover slip
8. Z2 Counter, ViCell analyser
9. Gilson pipette (P100)
10. Pipette filler

II. PROCEDURE

1. According to previous SOP, cells are trypsinised, neutralised and counted.

   Note: $2.5x10^5$ cells are needed per pellet culture.
2. 3 control cultures and 3 pellet cultures are set up to be harvested on day 21 (it is also possible to set up 3 cultures to be harvested on day 14).
3. The required number of cells for all the pellet cultures is placed into a tube and centrifuged at 100g for 5 min.
4. Suspension is then pelleted again at 100g for 5 min.
5. Cells are then resuspended in serum free chondrogenic media (0.5ml for Each chondrogenic pellet is required).

6. The tubes are centrifuged at 100g for 5 minutes.
7. The tubes are transported to a rack (within the incubator at 37°C, 5% CO_2, 90% humidity) and the caps are loosen to allow for gas exchange.
8. Medium is changed every other day by aspirating off as much medium as possible without disrupting the pellet and replacing with 500µl of fresh serum free chondrogenic medium.
9. After either 14 or 21 days in culture the cell pellets are harvested by aspirating off all medium and washing twice in D-PBS.
10. Pellets are allowed to air dry and then stored at -20°C until required for GAG measurement).

Adipogenic differentiation

I. MATERIALS AND EQUIPMENT

Solutions

1. 0.25% Trypsin-1mM EDTA
2. Complete hMSC serum free medium
3. Dulbecco's phosphate buffered saline (D-PBS)
4. Flasks of hMSC ready for subculture
5. Isopropyl alcohol (IPA)
6. serum-free adipogenic media - high glucose DMEM supplemented with ITS, 0.1 mM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM proline, Indomethacin 100mM (all from Sigma-Aldrich, USA) and 50–200 ng/ml BMP7 (PeproTech, USA)

Materials

1. BD PureCoat™ Amine 6-well culture plates
2. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
3. Sterile filter pipette tips (for P10, P100, P1000)
4. Sterile 15 ml centrifuge tubes
5. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Calculator and Timer
7. Haemocytometer with cover slip
8. Z2 Counter, ViCell analyser
9. Gilson pipette (P100)

10. Pipette filler

II. PROCEDURE

1. According to previous SOP, cells are trypsinised, neutralised and counted.
   Note: $2x10^5$ cells are seeded per well. 3 control wells and 3 adipogenic test wells are set up.
2. The required number of cells for 6 wells is placed into a 15ml tube.
3. The cells are diluted in complete hMSC serum free medium until the final volume is 12ml.
4. 2 ml of cell suspension are plated out per well in a 6 well plate.
5. Plates are placed in an incubator at 37ºC, 5% CO_2, 90% humidity.
6. After 24 hours cells should have adhered to the plastic surface of the plate.
7. Cells are allowed to grow until they reach confluence, media is changed every 3-4 days.
8. Once cells are confluent, 2ml of the adipogenic serum free media are added to the test wells. Control wells receive normal serum free growth medium.
9. Media is changed twice a week, ensuring the correct medium is added to each well.
10. Assay is harvested after 4 weeks depending on the condition of the monolayer.
11. Cells are fixed and Oil Red O staining is carried out.

E) xCELLigence MSCs profiling

I. MATERIALS AND EQUIPMENT

Solutions

1. 0.25% Trypsin-1mM EDTA
2. Complete hMSC serum free medium
3. Dulbecco's phosphate buffered saline (D-PBS)
4. Flasks of hMSC ready for subculture
5. Formaldehyde
6. Crystal violet solution
7. Isopropyl alcohol (IPA)

Materials

1. Sterile E-PLATE VIEW 16 (xCELLigence, ACEA, USA, ref. 06324738001)
2. Sterile serological pipettes (25 ml, 10 ml, 5 ml)
3. Sterile filter pipette tips (for P100)
4. Sterile 15 ml centrifuge tubes
5. Disposable container for liquid waste

Equipment

1. Biological safety cabinet (BSC)
2. 4°C refrigerator
3. Centrifuge
4. Incubator (37°C, 5% CO_2, 90% humidity)
5. Inverted microscope
6. Calculator and Timer
7. Haemocytometer with cover slip
8. Z2 Counter, ViCell analyser
9. Gilson pipette (P10, P100, P1000)
10. Multichannel Gilson pipette (P100)
11. Pipette filler

II. PROCEDURE

1. A few hours before beginning the procedure, 0.25% Trypsin-1mM EDTA is removed from the freezer, complete hMSC serum free medium from the 4oC refrigerator and D-PBS from room temperature storage and are allowed to come to room temperature.
2. The BSC is turned on and the interior work area is wiped down with alcohol.
3. Wiped containers with solutions at ambient temperature for a minimum of one and a half hours are placed in BSC.
4. Flasks with hMSC are removed from the incubator, wiped down with alcohol and placed in the BSC.
5. Medium from each flask is aspirated using a 25 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
6. 10 ml D-PBS is added to each T-175 flask using a 10 ml sterile serological pipette to wash the hMSC. (volumes are adjusted according to the volume of culture flasks)
7. D-PBS is aspirated using a 10 ml sterile serological pipette, without disturbing the monolayer, and discarded appropriately.
8. 5 ml 0.25% Trypsin – 1mM EDTA (alternatively Accutase or Accumax) is added to each flask using a 5ml sterile serological pipette. (volumes are adjusted according to the volume of culture flasks)
9. Flasks are incubated at room temperature for 5-8 minutes.
10. Cells are observed using an inverted light microscope to ensure that the hMSC have dislodged from the surface of the flask. If they are still adherent, flasks are incubated at room temperature until the hMSC are in suspension. Flasks are tapped sharply with gloved hand to ensure detachment.
11. The length of incubation time of hMSC in 0.25% Trypsin-1mM EDTA is recorded.

12. 0.25% Trypsin – 1mM EDTA is inactivated by adding 10 ml of prepared complete hMSC serum free medium without EGF and without ITS to each flask using a sterile serological pipette.
13. The suspension is pipetted over the bottom surface of the vessel several times to fully dislodge the hMSC, and then the cell suspension is transfered to a sterile 15 ml centrifuge tube. Suspension is centrifuged for 10 minutes at 900 g.
14. The interior work area of the BSC is wiped down with alcohol.
15. Centrifuge tubes are transfered from the centrifuge to the BSC. The supernatant is aspirated off and discarded in the container of bleach.
16. Cell pellets are resuspended and pooled from each centrifuge tube in a known volume of complete hMSC serum free medium supplemented with EGF and ITS (5-10 ml). The total volume of the final hMSC suspension is measured.
17. 50 µl aliquot of the hMSC cell suspension is removed for cell counting.
18. Cell number/ml is calculated by multiplying the number of cells counted in the 25 square grid by 104. Total cell number is calculated by multiplying the cell number/ ml by the total volume of the cell suspension.
19. According to the detailed well layout and schedule of the experiment 100 µl of hMSC serum free media are pipetted into each well, plates are inserted into the machine and step 1 is run. Note: Evaluate whether step 1 was successful.
20. After resuspending the cell pellet gently 90.000 cells are pipetted into labeled eppendorf tube and appropriate volume of cultivation media up to 1 ml total is added.
21. The test plate is put out of the xCELLigence machine. Note: Machine is waiting between steps 1 and 2, it is not necessary to press pause/stop button.
22. 10 µl of diluted cell suspension are pipetted into each well according to prepared layout (900 cells in each well). Note: Be careful when pipetting suspension - put tip under the media level /when viability of cells is lower then 95% pipette more cells (lower plating efficiency is anticipated when viability is low)/
23. Initial concentration 900 cells/well works for MSCs and DPSCs cultured without media exchange for 3-4 days (it is recommended to perform experiment in triplicates).

Note: When pipetting suspension still gently shake with the eppendorf tube! Be careful - cells in small drop sometimes adhere to lateral surfaces of the well (put the tip under the media level).

24. 90 µl of the media are added into each well and gently mixed.
25. The E-plates are transported back to the xCELLigence machine and next step will start either automatically or manually (it is recommend to measure every 15 minutes and to plan experiment for 4 days maximum without media exchange).
26. Following the cultivation and saving the data, the plate is removed from the xCELLigence machine. Note: First do software release, than hardware release.
27. In case the experiment is done in duplicates/triplicates - one of the wells should be trypsinized and used for cell counting and then for correlation of cell index and cell No.; in case of triplicates one well is stained for morphology assessment (we recommend standard Crystal Violet staining)
28. For DPSCs detachment we recommend 100 µl of Accutase (Invitrogen, USA), DPSCs are incubated for 10 min at, 37°C. After 10 min incubation cells are not inactivated, suspension in enzyme is directly counted either in hemocytometer or in ViCell analyzer. Note: Eevaluate cell detachment under the microscope!

Discussion

There has been relatively little progress in the development of new culture technologies for the large-scale manufacture of mesenchymal stem cells (MSCs). There is a strong possibility that this limited ability to produce stem cells will result in delays to the translation of new therapies to the clinic. The current state of the art has several weaknesses, in that, there are no standards for characterization, isolation or identification of MSCs from any tissue, nor are there standard protocols for differentiation of MSCs to various lineages. Additionally, surface markers used for MSCs characterization lack specificity and cryopreservation protocols are not standardized. Critically, current production methods for MSCs require the use of animal products with major contaminant implications. One of the goals of this thesis and PurStem project in parallel was to identify GMP-compliant raw materials, develop an Standard operating protocol for small scale serum-free media production, and validate analytical assays for use as Quality Control release assays for the production of GMP-compliant cells for clinical applications.

The method of classical preparation of the mesenchymal stem cells imposes substantive requirements on the purity of the environment. The expansion of mesenchymal stem cells starts with the resuspension of mononuclear cells from bone marrow blood in the culture medium in plastic or glass vessels. The mononuclear cells are then left to adhere to the surface of the vessels for 1-3 days. After this time, the non-adherent cells are removed and fresh medium is added to the adherent cells. The medium is usually changed twice a week (DiGirolamo et al., 1999)., Colter et al., 2000). During these manipulations, the cultivation vessels usually have to be opened, which leads to an increased risk of microbial contamination. After 2-3 weeks of cultivation, the bottom of the vessel is covered with 70-90% confluent mesenchymal cells monolayer, whereby from 7.5-10x10^6 bone marrow mononuclear cells it is possible to cultivate 0.4-1x10^6 mesenchymal cells. The classical environment for the cultivation of the MSC is the Dulbecco´s Modified Eagle Medium (DMEM) or the Eagle´s Minimal Essential Medium in alpha modification (alpha-MEM), supplemented with 10-20% fetal calf serum (Coelho et al., 2000, Novotová et al., 2003). The use of animal serum is at this time considered to be very problematic because of the possibility of animal disease transmission (e.g., bovine spongiform encephalopathy, BSE) and because of the possibility of severe allergic reactions to the animal protein, especially if the cells should be repeatedly administered to the same patient (Mackensen et al., 2000).

Using the classical method, as described above, it is not possible to get enough MSCs for the clinical use during a single expansion. Usually, the cells have to be passaged 1-2 times, which

increases the risk of the infectious contamination of the cell culture and also prolongs the time from the bone marrow harvest to the final product for the cellular treatment to 4-6 weeks. Apart from this, it appears that during the passaging, MSCs tend to lose their ability to differentiate into specialized tissues (Sugiura et al., 2004).

A number of researchers tried to resolve the above-described limitations, but none of them used a complex approach to the problem. As early as in 1995, Gronthos and Simmons (1995) have explored the effect of 25 recombinant growth factors on the growth of marrow stromal cells. They concluded that the highest numbers of CFU-F can be obtained with the combination of ascorbic acid, dexamethasone, platelet-derived growth factor BB (PDGF-BB) and epidermal growth factor (EGF). Jing-Xiang (2004) with co-workers have found that the 5 recombinant human monocyte colony stimulating factor increases the number of CFU-F by 25% and the total number of MSC eight- to tenfold. Tsutsumi (2001) with co-workers have shown that mesenchymal stem cells expanded with FGF-2 retain better differentiation ability when compared to MSCs expanded without this factor. These works have shown that the use of certain supplements or growth factors, most of which can be produced by recombinant technology, can lead both to a higher yield of mesenchymal stem cells and to the preservation of their ability to differentiate into specialized tissues. Other authors tried to overcome the need for fetal calf serum in the cultivation medium. Generally, the attempts to cultivate MSC in serum-free conditions were not successful (Hankey et al., 2001). Better, but ambiguous results were obtained with human serum or human plasma. In certain studies, the use of human autologous serum or autologous plasma led to better results than the fetal calf serum supplementation (Stute et al., 2004, Schecroun et al. 2004), but other studies led to different conclusions (Kuznetsov et al., 2000).

Why standardize MSC isolation, expansion, characterization and banking?

Adult human bone marrow derived mesenchymal stem cells (MSCs) hold great promise as clinical treatments for several pathologies due to their multipotentiality. However, given the low percentage of MSCs present in adult bone marrow compounded by the need for significant numbers of MSCs for clinical treatments, in vitro culturing of MSCs is necessary for clinical application. Currently there is no standardized approach for culturing adult human MSCs. Various combinations of basal medium, supplements and culture conditions can affect the phenotype of the cultured MSCs. Each laboratory has its own procedures for MSC culturing, making it difficult to compare published data between laboratories. There was therefore an obvious need for standardised best practice culture methods to be generated between PurStem laboratories, but eventually with translation to the field in general. For these reasons, one of the goals of PurStem

was to develop and perform inter-laboratory validation of standardised MSC isolation, expansion, characterisation and banking procedures to achieve the objectives of the PurStem project and to standardise the production of MSCs for clinical use.

Experiments looking at the culture process showed that it was relatively reproducible for all partner laboratories when using the standardised PurStem procedures. The amount of time for the number of cells to double did vary between partners and for different marrow samples. This variability could be explained to a certain extent by the relative number of cells prior to sub-culturing or by an overestimation of the number of MNCs at the start of culturing. Moreover, MSCs from all partners underwent osteogenesis, adipogenesis and chondrogenesis and there was no significant difference between partners.

A consistent phenotype (physical appearance and biochemical characteristics) is also important when isolating and culturing MSCs for therapeutic applications. Therefore, PurStem partners examined the phenotypes of MSCs isolated and cultured by each of the partner laboratories using the standardised PurStem procedures. This examination was carried out using fluorescence-activated cell sorting (FACS) analysis to measure markers expressed on the surface of the cells. The ensemble of surface markers expressed by MSCs is termed the MSC “Receptome”.

Pooling the data from each donor at all laboratories showed little variation in the highly expressed markers such as CD73, CD90, CD29. The strongest and most consistently expressed cell surface marker on the cultured cells from all of the partner laboratories was CD73, with all cells being > 90% positive compared to the isotype control. In agreement with the ISCT criteria and previously published data, the cultured cells from all partner laboratories were negative for the expression of CD19, CD45, CD34 and CD31. There is a degree of variation in the expression of CD105, CD146, CD13, CD106, with the most variable markers being CD105 and CD13. This is particularly relevant as the current “standard” of MSC phenotype, the position statement (Dominici et al., 2006) states that MSC must be positive for the expression of CD73, CD90 and CD105. Whilst a proportion of the cells cultured under standardised PurStem conditions did express CD105, the variable expression levels observed between laboratories in these standardised conditions indicates that this is not the most suitable marker for defining MSC phenotype and may in part contribute to the disparity of MSC phenotypes reported in the literature.

Our data indicates that the variation between the different donors is greater than the variation observed between the cells from the same donor being grown in different laboratories. This demonstrates the robustness of our standardised culture technique. The strongest and most consistently expressed cell surface marker on the cultured cells from all donors was CD73. The marker with the largest amount of variability in expression between different donors was CD13.

Expression levels of CD146 and CD105 were also found to be very variable between donors. Variations may be a reflection of the ‘in vitro’ age of the cultures, suggesting that cultures should be compared in terms of the number of population doublings (PD) rather than just passage number.

CD73, CD90, CD166 and CD81 showed no change in expression or variability, being consistently highly expressed at both passages tested. There was no change in the expression or variability for CD106, CD105, CD146 and CD13 at different passages, although all of these markers were highly variable between different donors. There was a trend for expression of CD271 to decrease with passage during culture, although the high donor variability means this was not statistically significant. Based on the two passages tested for cells cultured from the 3 donors, there were no statistically significant differences in the cell surface expression or variability observed between cells of different passages for any of the markers tested.

Can extended cultivation effect the MSCs “Receptome”?

Culturing can result in changes in MSCs that may impact their effectiveness for clinical therapies and can lead to a state where cells are no longer able to divide (senescence). Therefore, the MSC “Receptome” was characterised during long-term cultivation under standardised PurStem procedures. A statistically significant negative correlation was observed between the expression of Frizzled 9 (CD349) and TGFbR2 and the number of population doublings. SSEA4 expression showed a similar inverse but non-significant trend. Frizzled 4 (CD344) and TGFbR3 expression was consistently low. FGF receptors were expressed stably, but at relatively low levels. No significant trend was observed for CD106. These data suggest that the expression of Frizzled 9 may serve as the best potency indicator for MSCs cultured using standardised PurStem procedures.

The inter-laboratory variability of “Receptome” marker expression in cells was also studied using cells of varying passages cultured from 4 donors using standardised PurStem procedures. In spite of variation between donors, there was a overall trend of loss of Frizzled 9 expression during culture. There was variation in expression levels between different individuals but also between partners from the same sample. Therefore, Frizzled 9 expression may also be a useful measure of ‘culture quality’.

Additional receptors were examined to assess the degree of variation observed in marker expression between individual donors and between cells from the same donor cultured by different partners. Due to sample constraints, statistical analysis was not possible. However, following trends were observed. For some markers, such as FGFR1, there was a high degree of

variation between individual donors and for cells from the same donor cultured in different laboratories. The expression of other markers, including SSEA-4, CD106 and CD146, was highly dependent upon the culturing partner. This was surprising as the goal of standardising the isolation and culture procedures was to avoid culture-related variations. It is possible that seemingly minor differences, such as small variations in culture oxygen levels, may have a greater than expected influence

What are the requirements for GMP production of therapeutic MSCs?

PurStem initiative aimed to prepare for the advanced future manufacturing demand for MSCs in support of the industrialization of stem cell technology. To enable MSCs to be used in the clinic in a safe and efficient manner, all cell based medicinal products must comply with EU safety requirements and legislation. GMP requires that raw materials and other consumables used in the production and quality control analysis of products are of GMP grade to ensure the consistency of release results and patient safety. Standard operating procedures (SOPs) and validation of quality control release assays are also essential for GMP compliance with EU regulations.

In order to advance the bioprocessing of MSCs for clinical applications, initial in vivo studies were conducted using the MSCs isolated and cultured using the PurStem unified protocols. Furthermore, a list of the GMP compliant raw materials was identified and an SOP for the small-scale production of serum free media was written.

To comply with the GMP requirements we strived to cultivate MSCs in coating free environment. In 4 bone marrow samples freshly isolated MSCs were separated using Ficoll density gradient centrifugation and CD271+ immunomagnetic separation, then analyzed by flow cytometry and seeded in serum free media supplemented with EGF in special culture dishes (PureCoat Amine and Carboxyl, BD), then compared with directly plated MSCs. Following 14 days in culture, colonies were stained and duplicated samples were frozen for further qPCR analysis of telomere length. Directly plated MSCs were seeded in three different media (PS standard medium with 10% FCS, Serum-free medium supplemented with EGF and Human plasma+pHPL containing medium) and 3 different surfaces were tested to optimize serum-free cultivation protocol.

Why are serum-free culture conditions needed?

Clinical application of MSCs will require expansion and differentiation of primary MSCs in order to obtain sufficient therapeutic cell numbers as it is not possible to get enough MSC for the clinical use during a single expansion. Usually, the cells have to be passaged 1-2 times, which increases the risk of infectious contamination of the cell culture and also increases the time from the bone marrow harvest to the final production of the cellular treatment to 4-6 weeks.

Traditionally, MSC culture has been performed using bovine serum containing media. Bovine serum is the most widely used growth supplement for in vitro culture because of it possesses high levels of growth-stimulatory factors and low levels of growth-inhibitory factors. However, there are a number of risks associated with using animal derived products for the culture of therapeutic cells, including prion, viral or zoonose contamination (Mannello et al., 2007, Halme et al., 2006) and patient immune reaction (Tuschong et al., 2002, Selvaggi et al., 1997). In addition, there is a need for serum-free culture conditions for quality control of experiments between laboratories, as the variability of bovine serum can affect the reproducibility of results.

What are the current alternatives to animal-derived serum?

Human serum - An alternative to bovine serum albumin is autologous or allogeneic human serum. Autologous serum has proven to be as effective as bovine serum for the isolation and expansion of human MSCs (Stute et al., 2004), maintains higher cell motility compared to bovine derived serum (Kobayashi et al., 2005) and has eliminated the immune reaction observed with bovine serum cultured cells (Selvaggi et al., 1997 and Chachques et al., 2004). However, culturing MSCs in allogeneic human serum also results in growth arrest and cell death (Shahdadfar et al., 2005). Alternatively, fresh or frozen human plasma and platelets have been shown to be even more effective than bovine derived serum in maintaining human MSC proliferation (Muller et al., 2006). However, the use of human serum possess many of the same problems as bovine serum, such as variability in composition between donors and the collection of serum from patients with pathology, making the use of human serum an unattractive alternative to animal-derived serum.

Serum-free media - Unfortunately, the use of current serum-free conditions in culturing human MSCs selects for a subpopulation of cells that can survive serum deprivation and continue proliferating (Pochampally et al., 2004). A new complement of medium, growth factors, cytokines, etc. have to be developed specifically ensure consistent MSC cell surface receptor expression yielding uniform expansion of the MSC population in culture. Currently, there are commercially

available serum-free media. Mesencult-ACF from Stem Cell Technologies and Thera Peak from Lonza were tested during the study with unsatisfactory results.

PurStem approach - PurStem project set out to identify the MSC “receptome” and use this repertoire of growth factor receptors to develop novel serum-free media suitable for large-scale MSC production. Goals of this thesis and PurStem project were achieved by: developing and validating a collaborative, standardized procedure for the isolation, culture and cryopreservation of MSCs that produced consistent cell types even when grown in different laboratories; using the wealth of information obtained from characterisation of the MSC “receptome” to develop low and serum-free culture conditions where MSCs can survive and proliferate, thereby reducing or eliminating potential contamination issues associated with current serum-based culture methods.

Can human MSC differentiation be ensured in serum-free media?

During passaging, MSCs tend to lose their ability to differentiate into specialized tissues (Sugiura et al., 2004). As early as in 1995, Gronthos and Simmons (1995) have explored the effect of 25 recombinant growth factors on the growth of marrow stromal cells. The best results were obtained with the combination of ascorbic acid, dexamethasone, platelet-derived growth factor BB (PDGF-BB) and epidermal growth factor (EGF). Jing-Xiang et al. (2004) have found that the 5 recombinant human monocyte colony stimulating factor (rh M-CSF) increases the number of CFU-F by 25% and the total number of MSC eight- to tenfold. Tsutsumi et al. (2001) have shown that mesenchymal stem cells expanded with fibroblast growth factor 2 (FGF-2) retain better differentiation ability when compared to MSCs expanded without this factor. These works have shown that the use of certain supplements or growth factors, most of which can be produced by recombinant technology, can lead both to a higher yield of mesenchymal stem cells and to the preservation of their ability to differentiate into specialized tissues.

In our hands, the osteo- and chondrogenic potential of serum free expanded cells was compared with the potential of cells grown in serum containing media. MSCs cultured under the PurStem serum-free conditions show an elongated and fibroblast-like phenotype. Nonetheless, they maintain the ability to undergo osteogenic and chondrogenic differentiation.

Significant/Novel Results

1) Standardized protocols for MSC isolation, expansion, characterization and cryopreservation were generated. Standardized PurStem procedures yielded cells with a consistent cell surface phenotype, even in different laboratories.
2) CD73 is a much more robust positive MSC marker than CD105, which is often used in the literature.
3) CD13, CD146, CD106 are highly variable markers and therefore are not suitable for use as QC markers for defining MSCs.
4) Frizzled 9 may serve as the best potency indicator for MSCs cultured using standardized PurStem conditions.
5) Standardized serum-free protocols were generated for MSCs isolation, sub-culture, cryopreservation, differentiation assays and xCELLigence profiling.
6) MSCs cultured under PurStem serum-free conditions maintain their osteogenic and chondrogenic differentiation potential.
7) Serum-free culture methods are GMP compliant and suitable for the culture of stem cells for use in clinical applications.

Conclusions

Stem cells offer a promising avenue to therapy for a wide range of human diseases. However, for this potential to be realized, a consistent and plentiful supply of well-characterized stem cells is essential. To date, there has been relatively little progress in the development of new culture technologies for the large-scale manufacture of mesenchymal stem cells (MSCs). The current state-of-the-art has several weaknesses, in that, there are no standards for the characterization, isolation or identification of MSCs from any tissue, nor are there standard protocols for differentiation of MSCs to various lineages. Additionally, surface markers used for MSC characterization lack specificity and cryopreservation protocols are not standardized. Critically, current production methods for MSC require the use of animal products with major contaminant implications. Therefore, in an effort to overcome these issues, this thesis and PurStem project in parallel seek to identify the MSC "receptome" and use this repertoire of growth factor receptors to develop novel serum-free media for MSC production.

The objectives were to:

- Examine existing methods and approaches to the preparation of MSCs
- Identify current best practice
- Standardize the technology by means of a unified operating protocol.
- Develop new methods of culture and new media formulations by identifying the repertoire of growth factor receptors that exists on the surface of the cell.
- Use this information to develop new media using a combinatorial approach to the selection of growth factor supplements to modulate and optimize the growth kinetics and differentiation of the cells.
- Utilize recombinant human growth factors and so benefit from freedom of reliance on serum.
- Identify new reagents from this effort that will be used to characterize the cells.

As a first step to achieving these goals, all partners agreed on current best practice MSC methods. Consequently, a collaborative standardized procedure for the isolation, culture and cryopreservation of MSCs was developed and validated in each partner laboratory. Using FACS analysis, preliminary work on identifying the "in vivo" MSC transcriptome and changes due in vitro culture commenced. In parallel, the repertoire of growth factors expressed on the surface of the

MSC was identified. Furthermore, a list of novel antibodies for new surface antigens was characterised using a novel combination of transcriptional and biochemical approaches. With respect to the serum-free media formulation, preliminary studies examined MSCs viability in low serum conditions containing a number of growth factors and serum free conditions using growth factors in combination with various attachment factors. In order to advance the bioprocessing of MSCs for clinical applications, initial in vivo studies were conducted using the MSCs isolated and cultured using the PurStem unified protocols. Furthermore, a list of the GMP compliant raw materials was identified and an SOP for the small-scale production of serum free media was written.

A number of key results were achieved over the course of the project. Using the unified protocols for the isolation and characterization of MSC, initial results revealed that there was variability between partners at the start, but these variations reduced over time. Cumulative population doublings throughout the culture process from primary to the end of passage 2 culture indicated that the culture process was relatively reproducible between partner institutions. Moreover, using FACS analysis, it was revealed that the standardized PurStem culture conditions produced cells with a consistent cell surface phenotype even when grown in different laboratories. Using FACS and real time PCR, the repertoire of growth factor receptors and active transcription factors in the “in vivo” MSC was characterized; initial results revealed that cultured MSCs were a heterogeneous population of undifferentiated and more-committed, less proliferative cells, which evolve during passaging towards cellular senescence.

A wealth of information was generated by the surface receptome analysis, where a list of growth factor receptors expressed on the surface of the MSC was identified. The receptome was used to develop the serum free media. In a further effort to optimize the serum-free media formulation, a range of growth factor combinations were examined, and it was found that MSCs can attach, survive and proliferate in low serum and serum free conditions.

In the end, a rigorous protocol for the isolation and culture of MSCs has been developed and validated. The repertoire of identified growth factors was used to:

- Narrow down the growth factor combinations for the serum free media formulation,

- Provide information on new reagents that can strengthen the release criteria for MSCs,

- Help identify new methods for directly isolating MSCs from tissue based on immunoselection rather than adherence

- Help enable new methods for endogenous manipulation of MSCs.

The ability to produce GMP-grade human MSCs has advanced the state of the art in MSCs standards of preparation and release criteria. As a part of the PurStem project we have generated new standard operating procedures for the isolation and growth of MSCs, as well as new criteria that will be used to define the MSC for current applications in tissue engineering. These will contribute to the optimization of GMP manufacturing and banking of cells for use in clinical trials initially and ultimately as a commercial product.

Our effort has contributed not only to optimal stem cell manufacturing processes for Regenerative Medicine applications in the short-term, but has also advanced our basic understanding of MSC biology by defining the surface receptome. This database will improve our knowledge of the pathways that control mobilization and growth of the “in vivo” stem cell in response to injury, setting the stage for the development of next generation therapies which will exploit the self-repair potential of adult stem cells or stem cell targeting.

Since the onset of the PurStem project, a greater awareness of the research required to advance the large-scale manufacture of stem cells for clinical applications is becoming more apparent. In an effort to highlight the research currently underway in Europe, the PurStem website was created and has been constantly updated over the course of the project. Furthermore, PurStem has made an imprint on the world stage, with several conference presentations in the United States, United Kingdom, Ireland and the Czech Republic.

Moreover, to support the community, within this thesis I have organized seminars with patient groups and secondary schools students. Patient seminars were held where a distillation of the scientific work of the project was presented in addition to material focused on a particular audience or a particular medical condition. The audiences for these seminars included groups of individuals with a given condition (e.g. osteoarthritis), and also more mixed groups including carers and families of affected individuals. Namely, one large seminar for “Roska”, a non-profit patient association for people suffering from multiple sclerosis, was organized at Hradec Kralove on 22nd November 2010. This seminar focused on the potential value of stem cells as a therapy for multiple sclerosis.

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