Membrane vesicles

Piontek, Melissa Cäzilie; Roos, Wouter

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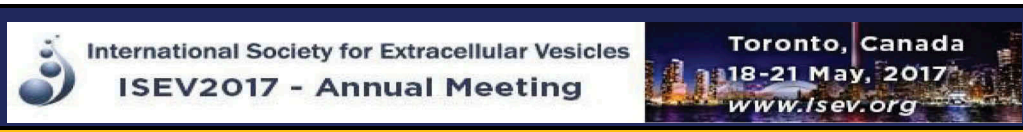

Wednesday, 17 May 2017 - Pre-Meeting Education Day			
8:30 - 5:30pm	**The Pre-meeting Education Day is a successful tradition of the annual ISEV meetings. Focusing on particular topics within the extracellular vesicle field, select experts provide deep insight into certain basic aspects of extracellular vesicles. These educational presentations collectively illuminate burning questions and provide a comprehensive insight into the topic.** ***Parallel Sessions*** ***Education Day First Sessions: Biology and Technology** ***Education Day Second Sessions: Extracellular Vesicles in Health and Disease**		
Thursday, 18 May 2017			
8:00 – 9:00am	Registration		
8:30 – 9:00am	Welcome Coffee		
9:00 – 9:20am	Opening Sessions (Room 1 Metropolitan Ballroom - West and Centre) Speaker: Philip Stahl, PhD		
9:30 – 10:30am	Plenary Session 1 –Extracellular Vesicles in Pathology of Complex Tissues Chairs: Andrew Hill, PhD; Susmita Sahoo, PhD		
10:30 – 11:00am	Networking Coffee		
11:00 – 12:30pm	**Symposium Session 1** **Therapeutic Applications of EVs** Room: Metropolitan Ballroom - West and Centre Chairs: Peter Quesenberry and Jan Lotvall	**Symposium Session 2** **Platelets, Coagulation, and Inflammation** Room: Metropolitan Ballroom - East Chairs: Eric Boilard and Pia Sijander	**Symposium Session 3** **EVs in Neurologic Diseases** Room: Harbour Ballroom Chairs: Lynn Pulliam and Laura Vella
12:30 – 1:30pm	Lunch		
1:30 – 2:15pm	**Symposium Session 4** **EV Biogenesis** Room: Metropolitan Ballroom - West and Centre Chairs: Matias Ostrowski and Crisslyn D'Souza	**Symposium Session 5** **EVs in Tumor Biology** Room: Metropolitan Ballroom - East Chairs: Michael Freeman and Carolina Soekmadji	**Symposium Session 6** **EVs in Inflammatory Diseases** Room: Harbour Ballroom Chairs: Edit Buzas and Rienk Nieuwland
2:20-3:00pm	**Oral with Poster Session 1** Room: Metropolitan Ballroom - West and Centre Chair: Thomas Kislinger	**Oral with Poster Session 2** Room: Metropolitan Ballroom - East Chair: Uta Erdbruegger	**Oral with Poster Session 3** Room: Harbour Ballroom Chair: Eric Boilard
3:00 – 3:30pm	Networking Coffee		
3:30-5:15pm	**Symposium Session 7** Emerging Technologies in EV CharacterizationRoom: Metropolitan Ballroom - West and Centre Chairs: Hubert Yin and John Nolan **Biotech Sponsored Session** 3:30 - 3:45pm	**Symposium Session 8** EV Interactions with Cellular Targets Room: Metropolitan Ballroom - East Chairs: Dolores Di Vizio and TBD	**Symposium Session 9** EV-Mediated Communication in Cancer I Room: Harbour Ballroom Chairs: Peter Kurre and Olga Volpert

5:15 – 6:30pm	Poster Session T01 - From Biogenesis to Targeting Poster Session T02 - EV Isolation Poster Session T03 - EVs in Tissue Protection and Repair Poster Session T04 - EVs in Cancer Therapy and Drug Resistance Poster Session T05 - Novel Developments in EV Characterization Poster Session T06 - Non-Cancer EV Biomarkers Poster Session T07 - EV Proteomics and Lipidomics Poster Session T08 - EVs in Viral and Bacterial Infections Poster Session T09 - EVs in Diseases of the Central Nervous System Poster Session T10 - EVs in Tumor Metastasis and Angiogenesis Poster Session T11 - EVs and the Immune System		
6:30 - 8:00pm	Satellite Event (Room: Metropolitan Ballroom - West and Centre) Meet the National and International Societies: *This event will provide an opportunity for scientific*		
Friday, 19 May 2017			
7:45 – 8:30am	Meet the Experts Session 1 EV-Mediated Functional Delivery of Protein and Nucleic Acids Room: Metropolitan Ballroom - West and Centre Moderator: Lucia Languino Speakers: Janusz Rak and Raghu Kalluri	Meet the Experts Session 2 EV Lipids and Lipidomics Room: Metropolitan Ballroom - East Moderator: Yong Song Gho Speakers: Hang Hubert Yin and Alicia Llorente	Meet the Experts Session 3 Rigor and Reproducibility in EV Analysis Room: Harbour Ballroom Moderator: Chris Gardiner Speakers: An Hendrix and Andreas Moller
9:00 – 10:00am	**Symposium Session 10** **Novel Developments in EV Isolation** Room: Metropolitan Ballroom - West and Centre Chairs: Alain Brisson and Dylan Burger	**Symposium Session 11** **EVs in Tumor Metastasis** Room: Metropolitan Ballroom - East Chairs: Lei Zheng and Yves DeClerck	**Symposium Session 12** **EVs in Viral Infections** Room: Harbour Ballroom Chairs: Marc-Andre Langlois and Caroline Gilbert
10:00 – 10:30am	Networking Coffee		
10:30 – 11:30am	Plenary Session 2 – Plasma Membrane and Cellular Vesicles (Room: Metropolitan Ballroom - West and Centre) Chairs: Xandra Breakefield; Alissa Weaver		
11:30 – 12:30pm	**Featured Abstracts** Room: Metropolitan Ballroom - West and Centre Chairs: Xandra Breakefield and Alissa Weaver		
12:30 – 1:30pm	Lunch		
1:30 – 3:00pm	**Symposium Session 13** **Novel Technologies in EV Characterization** Room: Metropolitan Ballroom - West and Centre Chairs: Joanne Lannigan and Rienk Nieuwland	**Symposium Session 14** **EVs in Cardiovascular Disorders** Room: Metropolitan Ballroom - East Chairs: Chantal Boulanger and Mike Davis	**Symposium Session 15** **EV RNAs as Cancer Biomarkers** Room: Harbour Ballroom Chairs: Andrew Hill and Kendall Jensen
3:00 – 3:30pm	Networking Coffee		
3:30 - 5:15 pm	**Symposium Session 16** **EV Omics** Room: Metropolitan Ballroom - West and Centre Chairs: Juan Falcon-Perez and Suresh Mathivanan	**Symposium Session 17** **EVs in Tissue Repair and Inflammation** Room: Metropolitan Ballroom - East Chairs: Chris Gardiner and Shilpa Buch	**Symposium Session 18** **Biogenesis: EVs and Viruses** Room: Harbour Ballroom Chairs: Leonid Margolis and Jennifer Jones

5:15 – 6:30pm	Poster Session F01 - EV-Based Cancer Biomarkers Poster Session F02 - EV Isolation: Developments Poster Session F03 - Bodyfluid Biomarkers of Cancer Poster Session F04 - EVs in the Tumor Microenvironment Poster Session F05 - Inflammatory Disorders, Tissue Injury, and Coagulation Poster Session F06 - EVs and Stem Cells I Poster Session F07 - EVs in the Central Nervous System Poster Session F08 - Intercellular and Inter-Organismal Crosstalk Poster Session F09 - EVs in Parasitic Diseases Poster Session F10 - EVs as Mediators of Cancer Cell Signaling Poster Session F11 - Cell and Tissue Remodeling and Repair		
6:30 – 8:00pm	Satellite Event (Room: Metropolitan Ballroom - West and Centre) ISEV-ISAC-ISTH Workshop: *This satellite event will provide insight into the scientific interaction of*		
Saturday, 20 May 2017			
7:45 – 8:30am	Meet the Experts Session 4 In Vivo Imaging-based Analysis of EV-biological Activity Room: Metropolitan Ballroom - West and Centre Chair: TBD Speakers: Takahiro Ochiya and Charles Lai	Meet the Experts Session 5 Vesicular and Non-vesicular Pathways of Extracellular RNA Release Room: Metropolitan Ballroom - East Chair: Esther Nolte-t Hoen Speakers: Alissa Weaver and Muneesh Tewari	Meet the Experts Session 6 EV-mediated Parasite-host Interactions Room: Harbour Ballroom Chair: Ana Claudia Torrecilhas Speakers: Rodrigo Soares and Martin Olivier
9:00 – 10:00am	**Symposium Session 19** **EVs in Tumor Immunity and Angiogenesis** Room: Metropolitan Ballroom - West and Centre Chairs: Carol Parent and Janusz Rak	**Symposium Session 20** **EVs in Stem Cell and Cardiovascular Biology** Room: Metropolitan Ballroom - East Chairs: Costanza Emanueli and Uta Erdbruegger	**Symposium Session 21** **Milk EVs** Room: Harbour Ballroom Chairs: Martinjn van Herwijnen and Patrick Provost
10:00 – 10:30am	Networking Coffee		
10:30 – 11:30am	Plenary Session 3 – From Extracellular Vesicles to Coordinated Behaviour of Cellular Populations (Room: Metropolitan Ballroom - West and Centre) Moderators: Marca Wauben; Ken Witwer		
11:30 – 12:30pm	ISEV General Assembly (Room: Metropolitan Ballroom - West and Centre)		
12:30 – 1:30pm	Lunch		**JEV Editorial Board Meeting**
1:30 – 3:00pm	**Symposium Session 22** **EV-Mediated Communication Between Host and Microorganisms** Room: Metropolitan Ballroom - West and Centre Chairs: Patricia Xander and Ana Claudia Torrecilhas	**Symposium Session 23** **EV-Based Cancer Biomarkers** Room: Metropolitan Ballroom - East Chairs: Aled Clayton and Lorraine O'Driscoll	**Symposium Session 24** **EV Functions in Inflammation** Room: Harbour Ballroom Chairs: Saara Laitinen and Takahiro Ochiya
3:00 – 3:30pm	Networking Coffee		
3:30-5:15	**Symposium Session 25** **EV-Mediated Communication in Cancer II** Room: Metropolitan Ballroom - West and Centre Chairs: Louise Laurent and Dave Carter	**Symposium Session 26** **EVs as Epigenetic Regulators** Room: Metropolitan Ballroom - East Chairs: Hidetoshi Tahara and TBD	**Symposium Session 27** **EVs in Cancer Progression and Therapy** Room: Harbour Ballroom Chairs: Andries Zijlstra and Peter Quesenberry

5:15 – 6:30pm	Poster Session S1 - EVs and Stem Cells II Poster Session S2 - EVs for Therapeutic Applications Poster Session S3 - EVs in Biofluids Poster Session S4 - Isolation, Characterization, and Detection of EVs Poster Session S5 - EVs in Cardiovascular Disease Poster Session S6 - EVs in Cancer Biology and Progression Poster Session S7 - Cancer Poster Session S8 - Viruses, Bacteria, Fungi, and Parasites		
8:00pm	Networking Event - **The ISEV2017 Networking Event provides a great opportunity to meet the leaders**		
Sunday, 21 May 2017			
7:45 – 8:30am	Experts Workshop Session 1 Workshop on exRNA biology and the analytical methods organized by the ERCC, NIH Room: Metropolitan Ballroom - West and Centre Moderator: Louise Laurent	Experts Workshop Session 2 EV-TRACK Room: Metropolitan Ballroom - East Moderator: An Hendrix	Experts Workshop Session 3 Demonstration Workshop Data Analysis with FunRich Room: Harbour Ballroom Moderator: Suresh Mathivanan
9:00 – 10:00am	**Symposium Session 28** **EVs in Cardiovascular Diseases and Vascular Disorders** Room: Metropolitan Ballroom - West and Centre Chairs: TBD and Jason Fish	**Symposium Session 29** **Evs in Immune System and Inflammation** Room: Metropolitan Ballroom - East Chairs: TBD and Eric Boilard	**Symposium Session 30** **Novel Developments in EV Biogensis and Characterization** Room: Harbour Ballroom Chairs: An Hendrix and Jeff Franklin
10:00 – 10:30am	Networking Coffee		
10:30 – 11:15am	Featured Abstracts Room: Metropolitan Ballroom - West and Centre Chairs: Andrew Hill, PhD; Susmita Sahoo, PhD		
11:20 – 11:50am	Wrap Up Sessions – Clinical (Room: Metropolitan Ballroom - West and Centre) Speaker: Uta Erdbrugger Wrap Up Sessions – Basic Science Speaker: Eric Boilard		
11:50-12:15pm	Special Achievement Award and Closing Remarks (Room: Metropolitan Ballroom - West and Centre)		

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Scientific Program ISEV2017
Thursday May 18, 2017

Room: Metropolitan Ballroom West and Centre
Opening Plenary Session 9:00–9:20 a.m.

Chairs: *Andrew Hill, PhD; Susmita Sahoo, PhD*

Speaker:
Philip Stahl, PhD (Washington University, St. Louis, MO, United States)
The Exosome Paradigm of Intercellular Communication

Room:
Plenary Session-1: Extracellular Vesicles in Pathology of Complex Tissues

Chairs: *Andrew Hill, PhD; Susmita Sahoo, PhD* 9:30–10:30 a.m.

Speakers:
Thomas Thum, PhD, MD (Hannover Medical School, Germany)
Non-coding RNA and Microvesicles in Cardiovascular Homeostasis and Disease
Jeffrey Wrana, PhD (Mt. Sinai Hospital, Toronto, Ontario, Canada)
The Role of Exosomes in Planar Cell Polarity in Pathological Cell Migration

Oral Sessions
Room: Metropolitan Ballroom West and Centre
Symposium Session 1 – Therapeutic Applications of EVs
Chairs: Peter Quesenberry and Jan Lotvall 11:00–12:30 p.m.

OT1.01

Therapeutic potential for Spodoptera-derived microvesicle delivery of the membrane transport proteins cystinosin, sialin and CFTR

Jodi Mullet and Jess Thoene

University of Michigan, MI, United States

Introduction: Therapeutic use of transmembrane proteins is limited because they irreversibly denature when away from their native lipid membrane. Mutations in such proteins cause many lethal disorders including two lysosomal transport disorders, cystinosis and infantile sialic acid storage disease due to defective cystinosin and sialin. Cystic fibrosis is due to mutations in the plasma membrane anion transporter CFTR. Cystinosin and sialin-deficient fibroblasts accumulate lysosomal cystine or sialic acid. Cystinosis patients develop highly painful corneal cystine crystals, currently treated hourly with cysteamine eye drops. We here show delivery of functional membrane transport proteins to fibroblasts and cornea via microvesicles.c
Methods: Transport proteins were cloned in Baculovirus and expressed in Sf9 cells at an MOI of 1.0. Microvesicles ~90 nm dia were purified from 3 d post lytic infection Sf9 supernatant, dialysed into Ham's F12 media, sterilised via 0.22 μm filters and 10^{11} microvesicles/ml (NanoSight quantitation) placed on cultured fibroblasts or ex vivo wt NZW rabbit ocular globes. Cystine and sialic acid were quantified by LC/MS and colorimetric assay, respectively. Cystinosin and sialin in the microvesicles was confirmed by protein LC/MS/MS (1). Delivery of cystinosin-GFP and GFP-CFTR to target tissue was determined by confocal immunofluorescence microscopy.
Results: We have previously shown that addition of cystinosin or sialin-containing microvesicles decreases stored lysosomal cystine or sialic acid by ~50% at 96 h and persists to 196 h after a single administration. No effect was seen on cells pre-loaded with 3[H] mannitol, precluding increased exocytosis (1). GFP-tagged transport proteins added to cultured normal or cystinotic fibroblasts or rabbit ocular globes displayed punctate perinuclear green fluorescence with time dependence and penetration of cystinosin-GFP into the cornea of ~50% after 96 h.
Summary: Use of microvesicles to deliver transmembrane proteins has significant potential to treat lethal inborn errors of transport at the lysosomal and plasma membrane. Cystinosin-containing microvesicle eye drops may be a significant advance by permitting weekly administration. Kickstart Award from the University of Michigan.

Reference

1. Thoene et al., *Mol. Gen. Metab.* 2013; 109: 77–85.

OT1.02

Exosome-mediated delivery of CFTR protein to human bronchial epithelia as a novel therapeutic strategy to treat Cystic Fibrosis

Inna Uliyakina[1], Justin Hean[1], Andreas Koschinski[1], Miguel Lobo[1], Samir El Andaloussi[1], Alison Mahoney[2], Ray Jupp[2] and Matthew J. Wood[3]

[1]University of Oxford, Oxford, United Kingdom; [2]UCB Pharma; [3]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom

Introduction: Cystic fibrosis (CF), the most common life-shortening genetic disorder among Caucasians, affects over 70,000 patients worldwide. CF is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, an anion (chloride/bicarbonate) channel that is expressed at the apical membrane of epithelial cells to control salt and water transport. To date more than 2000 mutations have been reported in the gene. For the majority of CF patients, successful therapy requires the replacement of the mutated gene or protein by a functional entity. As with many clinical trials for CF, gene therapies have been unsuccessful mainly due to the low uptake of CFTR cDNA through the thick mucus obstructing the airways and to the deleterious immune response of the host organism. Recently, exosomes have been demonstrated to efficiently and specifically deliver proteins, mRNA and si/miRNAs with little or no toxicity or immunogenicity *in vivo*. Here, we propose to use exosome-mediated delivery of CFTR protein to CF respiratory epithelia in order to restore the deficient chloride transport.
Methods: Exosomes were isolated by size-exclusion liquid chromatography and were analysed NTA, μBCA and western blot. Localisation and the plasma membrane (PM) stability of CFTR was monitored by live-cell confocal microscopy and cell-surface biotinylation, respectively. Functional activity of CFTR channel was measured by whole-cell patch clamp technique.
Results: In order to improve the trafficking of CFTR into exosomes, several fusion constructs containing CFTR and exosomal proteins were generated. For instance, CFTR was fused to exosomal membrane proteins such as tetraspanins, endosome- and exosome biogenesis-associated proteins. Fusion constructs were fully processed, expressed at the PM of the epithelial cells and functionally active as a chloride transporter. CF human bronchial epithelial cells depleted for CFTR protein were incubated with exosomes containing CFTR protein and the localisation of the exosome-delivered CFTR protein was monitored by confocal microscopy showing the successful uptake of the engineered exosomes.
Conclusions: Exosome-mediated delivery of CFTR is thus a promising solution to treat/alleviate CF pathology independently from the type of mutation.

OT1.03

Bio-inspired synthetic exosomes carrying microRNA let-7b for post-ischemic vascular regeneration

Sezin Aday[1], Inbal Halevy[2], Maryam Anwar[3], Marie Besnier[1], Cristina Beltrami[1], Andrew Herman[1], Susmita Sahoo[4], Enrico Petretto[5], Gianni Angelini[1], Dan Peer[2] and Costanza Emanueli[6]

[1]University of Bristol, Bristol, United Kingdom; [2]Tel Aviv University, Tel Aviv, Israel; [3]Imperial College London, London, UK; [4]Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai, New York, USA; [5]Duke-NUS Medical School, NC, USA; [6]Bristol Heart Institute, University of Bristol, Bristol, United Kingdom

Ischemic diseases are the leading cause of illness and death around the world. Localised therapeutic angiogenesis able to improve the microvascular network could help the suffering patients by providing additional blood flow to inadequately perfused areas. Exosomes with variable microRNA cargos are released from different progenitor cell types and stimulate angiogenesis in animal models. We recently showed that human pericardial fluid (PF) surrounding the heart also contains exosomes able to promote angiogenesis *via* the delivery of the microRNA let-7b-5p to recipient hypoxic endothelial cells (ECs). Here, we aimed to: (1) characterise the common microRNA cargo of endogenous angiogenic exosomes using bioinformatics, (2) exploit this knowledge to develop off-the-shelf artificial exosomes (AEs) with superior proangiogenic capacities, (3) validate the angiogenic potential of the bioinspired AEs. Pilot bioinformatics analyses integrating data of miRNA arrays on proangiogenic exosomes (from PF and bone marrow-derived $CD34^+$ cells) confirmed the enrichment of let-7b-5p in these exosomes. Next, we produced AEs containing either let-7b-5p or fluorescent cy5-cel-miR-39, as control. The AEs were uptaken by human ECs and pericytes cultured under hypoxic conditions, without causing toxicity. let-7b-AEs transferred functional let-7b, thus decreased the expression of *TGFBR1* and *CASP3* (validated targets of

let-7b-5p) in recipient cells. let-7b-AEs improved EC survival and angiogenesis *in vitro*. *In vivo*, cel-miR-39-AEs injection in ischemic murine muscles resulted in their uptake by 12% and 11% of the local microvascular ECs and pericytes, respectively. Overall, our preliminary results suggest the therapeutic potential of bioinspired AEs containing let-7b, which will be further developed by: (1) employing clustering techniques to find candidate miRNAs grouping with let-7b; (2) functionalising AEs to preferentially target ischemic ECs.

OT1.04

Scalable, cGMP-compatible purification of EV enriched with heterodimeric interleukin-15

Dionysios C. Watson[1], Bryant Yung[2], Aizea Morales-Kastresana[1], Cristina Bergamaschi[1], Bhabadeb Chowdhury[1], Jennifer C. Jones[3], Barbara Felber[1], Xiaoyuan Chen[2] and George Pavlakis[1]

[1]National Cancer Institute, National Institutes of Health, NY, USA; [2]National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, NY, USA; [3]National Cancer Institute, Vaccine Branch, MD, USA

Introduction: We previously showed that hollow-fibre bioreactors are a rich source of extracellular vesicles (EVs). These EVs were purified by ultracentrifugation; however, purification by ultracentrifugation was not easily scalable and preparations contained macromolecular contaminants. In this study, we tested scalable, cGMP-compatible purification methods to obtain highly purified preparations of EVs carrying heterodimeric interleukin-15 (hetIL-15), a cytokine tested in clinical trials for treatment of cancer.
Methods: We constructed a HEK293 cell line stably expressing a heterodimeric IL-15 /Lactadherin fusion protein. Cells were grown in a hollow-fibre bioreactor with serum-free media; conditioned media were clarified by centrifugation and filtration, and subsequently concentrated by tangential flow filtration (TFF). EVs were then purified by size-exclusion chromatography (SEC). EV preparations were characterised by nanoparticle tracking analysis (NTA), ELISA, flow cytometry, transmission electron microscopy (TEM) and mass spectrometry. Bioactivity of IL-15 was measured through the dose-dependent proliferation of the human NK-92 cell line upon exposure to the cytokine.
Results: Concentration by TFF (750kDa MWCO) removed many of the contaminating vesicle-free proteins. By monitoring 260/280 light absorption during SEC, the EV-containing fraction could be reliably collected, as confirmed by NTA. Particle yield of SEC was similar to that of ultracentrifugation, while purity (particle:protein ratio) was 8-fold higher. The major contaminant of ultracentrifugation, ferritin, was decreased by 26-fold by SEC. EV from cells expressing the hetIL-15/Lactadherin fusion protein contained 100-fold more cytokine compared to EV from cells expressing the natural cytokine, while both forms retained bioactivity.
Conclusion: Lactadherin fusion constructs remain EV-associated after SEC, and retain their bioactivity. Processing of bioreactor conditioned media by TFF ultrafiltration/concentration followed by SEC results in highly purified EV preparations. Given the scalability and cGMP compatibility of these methods, they could be useful in large-scale preparation of clinical grade EV.

OT1.05

Exosome-SIRPalpha, a CD47 blockade increases cancer cell phagocytosis

Eunee Koh[1], Yoosoo Yang[2] and In-San Kim[2]

[1]KU-KIST Graduate School of Converging Science and Technology, Seoul, Republic of Korea; [2]Korea Institute of Science and Technology, Seoul, Republic of Korea

CD47, a "don't eat me" signal, is over-expressed on the surface of most tumours that interacts with signal regulatory protein α (SIRPα) on phagocytic cells. By engaging SIRPα, CD47 limits the ability of macrophages to engulf tumour cells, which acts as a major phagocytic barrier. In this study, we developed an exosome-based immune checkpoint blockade that antagonises the interaction between CD47 and SIRPα. These exosomes harbouring SIRPα variants (SIRPα-exosomes) were sufficient to induce remarkably augmented tumour phagocytosis, lead to prime effective anti-tumour T cell response. Given that clustering of native CD47 provides a high binding avidity to ligate dimerised SIRPα on macrophage, nature-derived exosomes could be appreciable platform to antagonise CD47. Disruption of CD47-SIRPα interaction by SIRPα-exosomes leads to an increase in cells being engulfed by macrophages and a concomitant inhibition of tumour growth in tumour-bearing mice. Moreover, SIRPα-exosomes therapy promotes an intensive T cell infiltration in syngeneic mouse models of cancer, raising the possibility of CD47-targeted therapies to unleash both an innate and adaptive anti-tumour response. Note that very small amount of exosomal SIRPα proteins could effectively lead to phagocytic elimination of tumour cells both *in vitro* and *in vivo*. Our results suggest that superlative exosome-based platform has broad potential to maximise the therapeutic efficacy of membrane-associated protein therapeutics (1).

Reference

1. Koh et al., *Biomaterials* 2017; 121: 121–129.

OT1.06

Novel therapeutic strategies against cancer metastasis by targeting extracellular vesicles by specific antibodies

Nao Nishida-Aoki[1], Naoomi Tominaga[1], Fumitaka Takeshita[2], Hikaru Sonoda[1], Yusuke Yoshioka[1] and Takahiro Ochiya[1]

[1]Division of Molecular and Cellular Medicine, National Cancer Centre Research Institute, Japan; [2]Department of Functional Analysis, FIOC, National Cancer Centre Research Institute, Japan

Introduction: Cancer-derived extracellular vesicles (EVs) promote metastasis by forming cancer microenvironment and pre-metastatic niche. Therefore, inhibiting the pro-metastatic function of cancer-derived EVs is expected to suppress metastasis. We demonstrated the therapeutic concept of targeting EVs using an experimental model.
Methods: The antibodies specific to human CD9 and human CD63 were injected intravenously for every 3 days for a total of 3 times to an orthotropic mice model of highly-metastatic human breast cancer. After 35 days, the metastasis levels were evaluated by *ex vivo* imaging and immunohistochemistry. The EVs collected by ultracentrifugation from filtrated culture media were stained by a lipophilic dye PKH67 or DiR. To transiently remove mouse innate macrophages, clodronate liposomes were injected intravenously 5 days before the administration of the EVs.
Results: The species-specificity and the binding ability on the surface of the EVs from the human breast cancer cells of the antibodies were confirmed. Antibody treatment significantly reduced lung metastasis compared to the control IgG treatment. The antibodies did not decrease the size of the primary tumours, cell proliferation and invasion abilities, but decreased the amount of circulating cancer-derived EVs. These observations suggested that the antibodies suppressed metastasis by disrupting the EVs but not primary tumours. Indeed, the antibodies stimulated removal of EVs by macrophages both *in vitro* and *in vivo*. The stimulation of EV removal disappeared by depletion of innate macrophages of mice, indicating that the stimulation of removal of the EVs was macrophage-dependent.
Conclusion: Recognition of the cancer-derived EVs by antibodies suppressed lung metastasis, by stimulating the removal of the EVs by macrophages. Identifying the specific targets at the surface of the cancer-derived EVs is required for practical use.

Reference

1. Nishida-Aoki et al., *Mol. Ther.* 2017; 25: 181–191.

Room: Metropolitan Ballroom East
Symposium Session 2 – Platelets, Coagulation, and Inflammation
Chairs: Eric Boilard and Pia Sijander 11:00–12:30 p.m.

OT2.01

Extracellular vesicles from activated platelets: a quantitative cryo-electron microscopy and immuno-gold labelling study

Alain R. Brisson[1], Sisareuth Tan[1], Celine Gounou[1], Romain Linares[1], Nicolas Arraud[1] and Stephane Mornet[2]

[1]UMR-5248 CNRS – University of Bordeaux, Bordeaux, France; [2]UPR-ICMCB CNRS

Introduction: Upon activation, blood platelets release two types of extracellular vesicles (EV), namely microparticles characterised by the presence at their surface of phosphatidylserine (PS), which supports their role in haemostasis and in thrombosis (1), and exosomes characterised by their small size (50–100 nm) and the presence of CD63 on their surface (2). However, a clear distinction between microparticles and exosomes is hampered by the difficulty of EV characterisation, which results from their heterogeneity and from the lack of reliable methods allowing their isolation and quantification. Using cryo-electron microscopy (EM) and immuno-gold labelling (3), we have revisited the question of EVs released by activated platelets with the objective to provide a quantitative description of the size, phenotype and relative amounts of the main EV populations, focusing mainly on PS+ EVs CD41+ EVs and CD63+ EVs (4).
Methods: Peripheral blood was collected over citrate from four healthy adult donors after informed consent. Platelets from platelet rich plasma (PRP) samples were activated with thrombin, TRAP or CRP-XL. Gold nanoparticles conjugated with annexin-5, anti-CD41- or anti-CD63-mAbs were synthesised to label PS+ EVs, platelet-derived EVs and CD63+ EVs, respectively (3). Cryo-EM was performed as described in (3).
Results: We found that EVs activated by the three agonists presented a similar size distribution, about 50% of them ranging from 50 to 400 nm. About 60% EVs were found to expose CD41, a majority of them exposing also PS. Several mechanisms of EV formation are proposed to explain the presence of large amounts (40%) of CD41-negative or PS-negative EVs of large size, as well as large EVs containing organelles, principally mitochondria or granules.
We found also that the majority of EVs in activated platelets expose CD63. Two populations of CD63+ EVs were distinguished, namely large EVs with low labelling density and small EVs, likely the exosomes, with high labelling density.
Conclusion: This study provides a quantitative description of EVs from activated platelets and opens new insight on EV formation mechanisms.

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OT2.02

Morphological pathways involved in the release of extracellular vesicles from TRAP-activated platelets

Oumsalama K. Elhelu, Silvia De Paoli, Tseday Tegegn, Michael Strader, Ivan Tarandovskiy, Abdu Alayash, Mikhail Ovanesov and Jan Simak

FDA

Platelet-derived extracellular vesicles (PEVs), released upon platelet (PLT) activation play significant roles in inflammation, thrombosis, and other pathologies. Here we investigate PEV release from thrombin receptor-activating peptide-6 (TRAP-6)-activated washed PLTs. Two major PEV populations were isolated by a two-step centrifugation: 20,000*g* to collect the large and dense PEVs (L-PEVs), followed by 100,000*g* spin to obtain the small exosome size PEVs (S-PEVs). Orthogonal analysis of S-PEVs and L-PEVs by MS-proteomics, MS-lipid panel, electron microscopy (EM), laser-scanning confocal microscopy (LSCM), nanoparticle tracking analysis (NTA) and flow cytometry (FC) were used. Results indicate that about 90% of PEVs are in the size range 40–350 nm. S-PEVs compose the majority of the PLT vesiculome and have different proteomic and lipidomic profiles, compared to L-PEVs. Interestingly, S-PEVs have 2-fold higher phosphatidylserine content and corresponding 5.7-fold higher thrombin generation procoagulant activity per 1 nm^2 of the PEV surface area, compared to L-PEVs. FC analysis using MitoTracker and Tom20 Mab indicates that about 50% of FC-detectable PEVs contain mitochondria from which 10% refer to "free" mitochondria and 90% to mitochondria enclosed in vesicles. Based on MS-proteomics and extensive EM analysis, we propose four plausible mechanisms for PEV release: (1) plasma membrane budding, (2) extrusion of multi-vesicular bodies and cytoplasmic vacuoles, (3) plasma membrane blistering induced by PLT cytoskeleton contraction, and (4) we demonstrate a previously undescribed type of PEV – the "podiasomes" which originate from "pearling" of PLT pseudopodia. We show that the PLT vesiculome encompasses ectosomes, exosomes, free mitochondria, mitochondria-containing vesicles and exhausted PLT ghosts. The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination or policy.

OT2.03

Salivary EV: a new link between platelets and coagulation

Yuanjie Yu[1], Elmar Gool[2], René Berckmans[1], Auguste Sturk[1], Arjan Barendrecht[3], Coen Maas[3] and Rienk Nieuwland[1]

[1]Clinical Chemistry Department, Academisch Medisch Centrum; [2]Biomedical Engineering & Physics, Academisch Medisch Centrum; [3]UMC Utrecht

Human saliva contains extracellular vesicles (EV) exposing tissue factor (TF; (1)). This TF triggers coagulation in plasma, but whether salivary EV interact with platelets to promote coagulation is hitherto unexplored. We hypothesised that the TF-exposing EV from saliva interact with platelets exposing P-selectin, as described earlier for TF-exposing EV from tumour cells in a mouse model of vascular injury (2), making the platelets procoagulant. To test this hypothesis, we investigated the presence of P-selectin glycoprotein ligand-1 (PSGL-1) and CD24, two ligands of P-selectin, on salivary EV. PSGL-1 presence was below the detection limit when analysed by high-resolution flow cytometry. In contrast, the salivary EV abundantly expose CD24. Immune depletion of CD24-exposing EV completely removed the TF coagulant activity as measured by fibrin generation, and co-localisation of CD24 and TF on EV was confirmed by immunogold labelling transmission electron microscopy. In a whole blood flow model, salivary EV accumulated on the surface of aggregated platelets and the deposited EV promoted fibrin generation . Whether the deposition of EV is indeed mediated by the interaction between CD24 and P-selectin, is currently being studied. Collectively, we demonstrate that $CD24^+/TF^+$ EV may be a novel link between platelets and coagulation at a site of vascular injury.

References

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OT2.04

Pregnancy-associated circulating extracellular vesicles induce different phenotype changes in monocyte and trophoblast cell lines

Árpád Ferenc Kovács[1], Nóra Fekete[1], Orsolya Láng[1], László Kőhidai[1], Lilla Turiák[2], Rigó János[3], Edit Buzás[1] and Éva Pállinger[1]

[1]Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary; [2]Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary; [3]1st Department of Obstetrics and Gynecology, Semmelweis University, Budapest, Hungary

Introduction: Extracellular vesicles (EVs) represent a critically important, directed intercellular communication pathway during pregnancy. Circulating EVs in different microenvironments may exert divergent effects on the potential target cells. The aim of our study was to assess the effects of preeclampsia-associated circulating EVs (PE-EV) on monocytes and trophoblast cells.
Methods: BeWo trophoblast and THP-1 monocyte cell lines were used as model systems. EV-enriched preparations from blood plasma of healthy and preeclamptic third trimester pregnant women were isolated by differential centrifugation and were characterised by flow cytometry, DLS, TEM. The protein and miRNA cargos of EVs were assessed by mass spectrometry and a PCR Array, respectively. We evaluated the binding of EVs and the EV-induced cellular changes by flow cytometry. Changes in the expression of genes encoding for inflammatory and adhesion molecules were quantified by RT-PCR. Time dependent, EV-induced cytokine production was evaluated by a cytometric bead assay and a protein array. We used healthy pregnant-derived EVs (HP-EV) as biological controls.
Results: Circulating EVs bound onto both cell lines, however, they induced differential phenotypic changes. THP-1 cells produced significantly more inflammatory cytokines (TNF, IL-6 and IFN gamma) upon PE-EV treatment than upon treatment with HP-EVs. Analysis of proteins showed that preeclamptic EVs carried more proteins involved in biological processes related to inflammation, cell migration and adhesion as compared to HP-EVs.
Conclusion: The possible systemic effects of EVs exerted on monocytes and locally, on pregnancy-specific trophoblast cells were reflected by the high number of differential changes induced by the circulating EVs in these cell types. Gene expression, cell surface protein- and secreted cytokine patterns were all differentially influenced by PE-EVs. Circulating PE-EVs modified monocyte and trophoblast functions in a complex manner, suggesting that they might participate in the pathogenesis of preeclampsia.

OT2.05

Interaction of microvesicles with immune cells in lipopolysaccharide-stimulated whole blood

Rene Weiss[1], Marion Gröger[2], Sabine Rauscher[2], Birgit Fendl[1], Michael B. Fischer[3], Viktoria Weber[1] and Andreas Spittler[4]

[1]CD-Laboratory for Innovative Therapy Approaches in Sepsis; [2]Core Facility Imaging; [3]Department for Health Science and Biomedicine; [4]Core Facility Flow Cytometry & Surgical Research Laboratories

Introduction: Cells stimulated with lipopolysaccharide (LPS) produce extracellular vesicles. Here, we aimed to study the release of microvesicles (MVs) in LPS-stimulated whole blood and their interaction with immune cells.
Methods: Freshly drawn whole blood anticoagulated with heparin was stimulated with LPS from *E. coli* (0.1 µg/ml; 3 h; gentle rolling; 37°C) or left untreated. Cell-MV interaction was characterised and visualised using imaging flow cytometry (ImageStream^x MkII, Millipore). Leukocyte populations, free MVs, and cell-bound MVs were determined after incubation of whole blood with antibodies against CD45, CD14, CD16, CD15, CD3, CD56, CD235a and CD41, as well as with lactadherin (LA) as marker for phosphatidylserine-exposing MVs. Whole blood was diluted 1:10 with phosphate buffered saline prior to analysis. Cell populations were additionally sorted (Moflo Astrios EQ, Beckman Coulter) and subsequently visualised using confocal microscopy (LSM780 Airyscan, Zeiss). Whole blood was centrifuged two times (2500 g, 10 min; 13,000 g, 15 min, both at room temperature), and free MVs were characterised in platelet free plasma (PFP) using flow cytometry (CytoFLEX, Beckman Coulter). Triggering signal for MVs analysis was set to FITC conjugated lactadherin.
Results: In LPS-stimulated whole blood, a higher percentage of monocyte-MV aggregates ($CD14^{++}LA^{+}CD41^{+}$, 99% *vs.* 88%), granulocyte-MV aggregates ($CD15^{low}LA^{+}CD41^{+}$, 60% *vs.* 24%), NK cell-MV aggregates (7% *vs.* 0%) as well as T-cell-MV aggregates (4% *vs.* 1%) were present as compared to the unstimulated control. No MVs double positive for LA and antigens other than CD41 were detected on leukocytes. There was no substantial difference in counts of free MVs in LPS-stimulated and unstimulated samples (18,876 ± 6,125 MVs/µl *vs.* 17,191 ± 3,618 MVs/µl).
Conclusion: Imaging flow cytometry is a suitable method to study the interaction of extracellular vesicles with their target cells in whole blood. Platelet derived vesicles adhere preferentially to monocytes and granulocytes, while almost no MVs are bound to T-cells and NK cells.

OT2.06

Lymph as a vector of microparticles during rheumatoid arthritis

Nicolas Tessandier[1], Imene Melki[1], Nathalie Cloutier[1], Andreea Milasan[2], Catherine Martel[2], Paul R. Fortin[1] and Eric Boilard[3]

[1]CRCHU de Québec – Université Laval, Ville de Quebec, Canada; [2]Montreal Heart Institute – Department of Medicine, Faculty of Medicine, Université de Montreal, Montreal, Canada; [3]Department of Microbiology-Infectious Disease and Immunity and Faculty of Medicine, Université Laval, Ville de Quebec, Canada

Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease leading to progressive damage of bone and cartilage. Different players are implicated in the pathogenesis, but the role of immune complexes (ICs) is recognised as critical. While the joint represents the major site of the inflammatory process, extra-articular manifestations are also involved. During RA, the synovial fluid is enriched in leukocytes inflammatory molecules, and platelet-derived microparticles (PMP), which are suggested to amplify inflammation. Lymphatic circulation drains plasma ultrafiltrate of the interstitial medium through lymphatic capillaries and vessels to the heart. We hypothesise that in RA, lymph draining the inflamed joints could propagate PMP to extra-articular sites.
Methods: We used the K/BxN serum transfer murine model of RA. As FcγRIIA is the sole receptor for IC on human platelets, and mouse platelets are devoid of any receptor for immunoglobulin G IC, we further considered the contribution of IC in the process using transgenic mice expressing FcγRIIA on all myeloid cells, including platelets. We collected lymph from control and RA mice and ensured limited blood contamination along the process by quantifying erythrocytes and platelets. We analysed MP composition by cryo-electro-microscopy, dynamic light scattering and flow cytometry.
Results: We reveal the presence of MPs (average diameter 150 nm) in lymph, the majority of them not exposing phosphatidylserine. Among identifiable MPs, PMPs dominantly accumulate in lymph. Of interest, concentrations of PMPs increase significantly during RA, dependently of FcγRIIA signalling, specifically in the lymph draining the inflamed articulation and not in lymph draining another anatomical site.
Conclusion: Since PMPs are known to participate in cellular communication, their increased concentrations during RA open up new horizons to understand the consequences of extravasated MPs during a sustained inflammation.

Room: Harbour Ballroom
Symposium Session 3 – EVs in Neurological Diseases
Chairs: Lynn Pulliam and Laura Vella 11:00–12:30 p.m.

OT3.01

Microglia release distinct extracellular vesicle populations in response to different pathological stimuli

Pia Pužar Dominkuš[1], Matjaž Stenovec[2], Jure Loboda[1], Simona Sitar[3], Nataša Resnik[4], Saša Trkov Bobnar[2], Eva Lasič[2], Ana Plemenitaš[5], B. Matija Peterlin[6], Peter Veranič[4], Marko Kreft[2], Ema Žagar[3] and Metka Lenassi[1]

[1]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; [2]Laboratory of Neuroendocrinology-Molecular Cell Physiology, Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; [3]Department of Polymer Chemistry and Technology, National Institute of Chemistry, Ljubljana, Slovenia; [4]Institute of Cellular Biology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; [5]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia; [6]Department of Medicine, University of California San Francisco, USA

Introduction: Microglia protect the central nervous system against injury or infection, but also promote neurodegeneration when activated improperly. Activated microglia may communicate with the environment by the release of extracellular vesicles (EVs). We here examined whether different pathological stimuli (ATP – a signal for brain lesion, Ca^{2+} ionophore ionomycin and expression of HIV-1 protein Nef) evoke release of distinct EVs compared to resting immortalised human microglia.
Methods: We analysed morphology and molecular composition of EVs by transmission electron microscopy, asymmetric-flow field-flow fractionation connected to various detectors (optimised for detection of the entire range of EV sizes), flow cytometry, nanoparticle tracking analysis and immunoblotting; and examined the properties of punctuated Nef.GFP in live cells by confocal microscopy.
Results: The average radius (R_{rms}) of EVs constitutively released from non-stimulated microglia (~5×10^7 EVs/10^6 cells) increased from 191 nm (after 24 h incubation) to 365 nm (48 h) and 445 nm (72 h). After pulse (30 min) increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), bigger (R_{rms} 338 nm (ATP), 422 nm (ionomycin)), but not more numerous EVs with specific protein composition, were released (24 h). Conversely, EVs released from Nef.GFP-expressing cells (48 h) were more concentrated (up to 30×), smaller (R_{rms} 172 nm), floated on sucrose gradient in exosome fractions (immuno-positive for flotillin, Tsg101, annexin) and contained Nef.GFP to a small extent. Nef was also released with flotillin-positive EVs from HIV-1 infected microglia. In live cells, punctuated Nef.GFP comprised large, $[Ca^{2+}]_i$ independent, non-directional population that differed from the dextran- and LysoTracker-labelled vesicles; mobility of later was diminished in Nef.GFP-expressing cells in comparison to controls.
Conclusion: Microglia respond to diverse pathological stimuli by releasing specific (but still heterogeneous) EV populations, which could explain diverse functions of microglial EVs.

OT3.02

Serum miRNA exosomal biomarkers associated with Alzheimer's disease are also detected in brain derived exosomes from Alzheimer's human post-mortem tissue

Lesley Cheng[1], Laura J. Vella[2], Benjamin J. Scicluna[1], Colin L. Masters[2], Malcolm Horne[2], Kevin J. Barnham[2] and Andrew F. Hill[1]

[1]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Victoria, Australia; [2]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Victoria, Australia

Introduction: Alzheimer's disease (AD) affects more than 55 million people worldwide and is expected to double every 20 years in the absence of disease-modifying drugs. Therapeutic strategies aimed at limiting neurodegeneration require methods to diagnose the disease in preclinical patients. Several blood-based tests have been explored to detect AD however, evidence is required to determine whether blood sampling is an appropriate specimen to diagnose brain diseases. Previously we isolated serum exosomes from AD patients which displayed an abnormal composition of 16 specific microRNA (miRNA) biomarkers compared to controls.
Methods: To provide evidence that our serum exosomal miRNA biomarkers are suitable for the detection of a brain condition, we also profiled exosomes isolated from *post-mortem* human AD (n = 8) and control (n = 8) brain tissues. Exosomes were extensively characterised to meet the minimal experimental requirements set out by The International Society for Extracellular Vesicles to be defined as exosomes and small RNA profiling was performed by next-generation sequencing.
Results: Brain derived exosomes (BDEs) were found to contain a unique profile of small RNA, including miRNA, compared to whole tissue. Furthermore, all 16 AD serum exosomal biomarkers, identified in our previous study, were detected in BDEs including a panel of BDE specific miRNA that target genes involved in AD pathology. These genes were then validated by qRT-PCR in human tissues and translated to AD cell models with the aim to use mimetic exosomes loaded with miRNA to counteract imbalances of mRNA transcription.
Conclusion: This work has identified a highly specific panel of miRNA that is both present in the brain and blood of AD patients. The miRNA candidates can be used to develop a blood-based diagnostic test highly relevant to a brain disease, equivalent to non-invasive brain biopsy, and further studied to understand AD pathology and other neurodegenerative diseases to identify therapeutic targets.

OT3.03

Neurons export extracellular vesicles enriched in molecular chaperones and misfolded proteins

Jingti Deng and Janice E. A. Braun

University of Calgary, Calgary, Canada

Aims: The transmission of misfolded/toxic proteins, such as tau, superoxide dismutase 1, α-synuclein or huntingtin from affected to unaffected areas of the brain is a hallmark of many neurodegenerative diseases. The differences between the pathogenic transmission of toxic proteins and the routine export of extracellular vesicles that mediate the transfer of hydrophobic and cytosolic proteins, lipid and RNA between cells is not clearly defined. To address this knowledge gap, we have chosen to investigate the impact of molecular chaperones on the export of cellular proteins. Many molecular chaperones contribute to proteostasis, and we have focused on the J protein co-chaperone family that is known to selectively target client proteins. Cysteine string protein (CSPα) is a critical neural J protein and we have recently demonstrated that it is exported from neurons in extracellular vesicles.
Methods: Extracellular vesicles were isolated from mouse brain slices as well as CAD cells transiently expressing either the polyglutamine expanded protein 72Q huntingtin^{exon1} or superoxide dismutase-1 (SOD-1^{G93A}), along with select J proteins. The protein content of extracellular vesicles was determined by western blot analysis.
Results: Here we show that exported vesicles from native mouse neurons contain J protein co-chaperones, in particular, CSPα. In CAD cells expressing disease-associated proteins, such as 72Q huntingtin^{exon1} or SOD-1^{G93A} but not CSPα, these toxic proteins are retained by the cell. However, in the presence of wild-type CSPα, but not the CSPa mutant $^{HPD/AAA}$, both 72Q huntingtin^{exon1} and SOD-1^{G93A} are transported out of the cells via extracellular vesicles. The vesicle-based export of various other proteins (e.g. Gas) is unaffected by WT CSPa.
Conclusions: Our results indicate that J proteins export specific proteins via extracellular vesicles. These findings suggest a link between the CSPα-mediated removal of toxic proteins and the transmission of misfolded/toxic proteins from affected to unaffected areas of the brain.
Funding: This work was funded by the Alberta Prion Research Institute.

OT3.04

Exosomal microRNAs in cerebrospinal fluid of patients with genetic frontotemporal dementia in the genetic frontotemporal dementia initiative – a biomarker study

Raphael Schneider[1], Paul McKeever[1], TaeHyung Kim[2], Caroline Graff[3], John van Swieten[4], Jonathan Rohrer[5], Robert Jr Laforce[6], Daniela Galimberli[7], Mario Masellis[8], Zhaolei Zhang[9], Janice Robertson[10] and Carmela Tartaglia[10]

[1]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada; [2]Department of Computer Science, University of Toronto, Toronto, Canada; [3]Department of Neurobiology, Karolinska Institute, Stockholm, Sweden; [4]Department of Neurology, Erasmus Medical Centre, Rotterdam, The Netherlands; [5]Dementia Research Centre, University College London, London, United Kingdom; [6]Département des Sciences Neurologiques, Université Laval, Quebec City, Canada; [7]Department of Physiopathology and Transplantation, University of Milan, Milan, Italy; [8]LC Campbell Cognitive Neurology Research Unit, University of Toronto, Toronto, Canada; [9]The Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Canada; [10]Tanz Centre for Research in Neurodegenerative Disease, University of Toronto, Toronto, Canada

Introduction: The lack of biomarkers for frontotemporal dementia (FTD) results in diagnostic delays and hinders drug development. Hence, there is an urgent need for diagnostic biomarkers. Investigating genetic FTD provides the opportunity to study pre-symptomatic individuals who are at increased risk of developing the disease. MicroRNAs can regulate mRNAs in disease pathways and have remarkable potential as biomarkers. Due to vesicular protection in exosomes, microRNAs are relatively stable in body fluids. To determine whether exosomal microRNAs in cerebrospinal fluid of patients with FTD can serve as diagnostic biomarkers, we characterised exosomal microRNA expression in pre-symptomatic and symptomatic individuals carrying a pathogenic mutation.
Methods: We recruited participants to this multicentre study who either were known carriers of a pathogenic mutation or were at risk of carrying a mutation because a first-degree relative was a known symptomatic mutation carrier. We isolated exosomes from cerebrospinal fluid using a commercially available kit. MicroRNA extraction was followed by real-time polymerase chain reaction in 384-well plates containing a total of 752 human microRNA primers. Exosomal microRNA expression was assessed in 23 pre-symptomatic and 17 symptomatic mutation carriers.
Results: MiR-204-5p and miR-632 were significantly decreased in symptomatic compared to pre-symptomatic mutation carriers ($p < 0.005$). Decrease of miR-204-5p and miR-632 revealed receiver operator characteristics with an area of 0.89 [confidence interval of 0.80–0.99] ($p < 0.05$) with potential for a diagnostic biomarker. Let-7a-5p, miR-23b-3p, miR-29a-3p, miR-30b-5p, miR-605-5p, and miR-892a were found less commonly in symptomatic compared to pre-symptomatic mutation carriers and healthy non-mutation carriers ($p < 0.05$). MRNAs targeted by these microRNAs were found in pathways of neurodegeneration.
Conclusion: Decrease of certain exosomal miRNAs has potential as diagnostic biomarker for FTD. Validation of our results in independent patient cohorts including sporadic cases will be necessary before this test can be applied in clinical practice.
This work was submitted by MC Tartaglia, on behalf of the Genetic FTD Initiative, GENFI

OT3.05

The novel long non-coding RNA TALNEC2 regulates the stemness and mesenchymal transformation of glioma stem cells and their exosome-mediated interaction with microglia cells

Shlomit Brodie[1], Simona Cazacu[2], Laila Poisson[2], Steve Kalkanis[2], Doron Ginsburg[3] and Chaya Brodie[4]

[1]Bar-Ilan University, Israel; [2]Henry Ford Health Systems, Detroit, MI, USA; [3]Faculty of Life Sciences, Bar-Ilan University, Israel; [4]Faculty of Life Sciences Bar-Ilan University, Israel and Neurosurgery Department, Henry Ford Health Systems, Detroit, MI, USA

Glioblastoma (GBM) are characterised by an infiltrative nature and high resistance to radio- and chemotherapy. GBM contain a subpopulation of glioma stem cells (GSCs) that is implicated in therapy resistance and tumour recurrence. Long non-coding RNAs (lncRNAs) play major roles in various processes associated with tumorigenesis and stemness. Here, we report the expression and functions of a novel lncRNA, TALNEC2 that was identified using RNA seq of E2F1-regulated lncRNAs. TALNEC2 expression was increased in astrocytic tumours in a grade-dependent manner and in mesenchymal GBM compared with the proneural and G-CIMP subtypes. Moreover, TLANEC2 was more significantly expressed in GBM specimens derived from short-term (<9 months) compared to long-term (>3 years) survivors. TALNEC2 was not expressed in normal brain tissues, astrocytes or neural stem cells, but its expression was high in GSCs and glioma cell lines. Silencing of TALNEC2 resulted in a decrease in the self-renewal of GSCs, expression of stemness and mesenchymal markers and in increased sensitivity of GSCs to radiation (3 Gy). Moreover, silencing of TALNEC2 resulted in inhibition of xenograft growth and prolonged animal survival. Using miRNA sequencing we identified specific miRNAs that were altered in the silenced cells and that mediated TALNEC2 effects via targeting of NF-kB, SOX2 and Dicer pathways. TALNEC2 was highly enriched in exosomes secreted from GSCs and played a role in the interaction of GSCs with microglia and in their polarisation by altering the delivery of miR-21 and miR-195 to these cells. Moreover, TALNEC2 was detected in serum exosomes of mice bearing GSC-derived xenografts. In conclusion, we identified a novel E2F1-regulated lncRNA that induced mesenchymal transformation and stemness of GSCs. The expression of TALNEC2 is associated with the increased tumorigenic potential of GSCs, their resistance to radiation and with the cross talk of GSCs and microglia. We conclude that TALNEC2 is an attractive therapeutic target for the targeting of GSCs and the treatment of GBM.

OT3.06

Neuronal exophers: a novel large vesicle that functions in the removal of neurotoxic cytoplasm components

Ilija Melentijevic[1], Marton Toth[1], Meghan Arnold[1], Ryan Guasp[1], Girish Harinath[1], Ken Nguyen[2], Daniel Taub[3], Alex Parker[4], Christian Neri[5], Christopher Gabel[3], David Hall[2] and Monica Driscoll[1]

[1]Rutgers, The State University of New Jersey, USA; [2]Albert Einstein College of Medicine; [3]Boston University Medical Campus, MA, USA; [4]Université de Montreal, Montreal, Canada; [5]Institut de Biologie Paris-Seine (IBPS), CNRS UMR 8256, Paris, France

Combating late-onset neurodegenerative disease and age associated functional decline in brain are major health challenges of our time. For the effective design of interventions that protect the nervous system from disease-induced and/or age-associated deterioration, we must fully understand endogenous mechanisms for neuronal protection and how they might fail to enable disease promotion. Recently, it has come to be appreciated that neurodegenerative disease proteins/aggregates can be found outside of mammalian neurons, and when outside can actually be taken up by neighbouring cells. Transfer of offending molecules has been suggested to be a mechanism of pathogenesis spread for multiple neurodegenerative diseases, including the prevalent Alzheimer's and Parkinson diseases. We discovered a novel capacity of young adult *C. elegans* neurons – neurons can extrude large (~4 μM) vesicles, which can include aggregated human neurodegenerative disease proteins, mitochondria or lysosomes, but no nuclear DNA. We call these extrusions "exophers". The ability to jettison cell contents is first evident about day 2–3 of adult life, coincident with the documented changes to adult proteostasis, and is then minimal until 9–10 days of adult life. Extrusion is increased when protein turnover or autophagy is inhibited. Moreover, exophers can selectively incorporate aggregation-prone proteins and oxidised mitochondria. Exopher contents can appear later in remote cells. Neurons that have made exophers appear to maintain functionality longer than non-exopher producing neurons. Thus, this pathway may constitute a novel neuronal protection mechanism that serves to maintain protein/organelle homeostasis when other systems are compromised. We propose that the neuronal extrusion phenomenon constitutes a significant but currently unknown conserved pathway by which healthy neurons maintain their functions, and speculate that, in diseases, this pathway may malfunction to promote spread of pathology. We will present the basic characterisation of neuronal exopher production and our latest data on genetic influences on exopher generation.

Room: Metropolitan Ballroom West and Centre
Symposium Session 4 – EV Biogenesis
Chairs: Matias Ostrowski and Crislyn D'Souza-Schorey 1:30–2:15 p.m.

OT4.01

Terminal complement components are critical in the release of cellular RNA in circulation

Virginia Camacho[1,2], John Tigges[1], Shulin Lu[1], Thomas Thomou[1], Vasilis Toxavidis[1], Horea Rus[3] and Ionita C. Ghiran[1,2]

[1]Beth Israel Deaconess Medical Center; [2]Harvard Medical School, MA, USA; [3]University of Maryland, MD, USA

Introduction: Despite of over 10 years of intense research, the intimate mechanisms responsible for extracellular vesicles (EVs) formation (exosomes and microvesicles), and the release of cellular RNA species (exRNAs) in circulation are currently unknown. The complement system is comprised of over 20 soluble and membrane bound proteins with critical roles in recognising, binding, and removal of foreign particles as well as initiating and regulating innate and acquired immune responses. Activation of the complement system occurs during both, normal (*circadian* variation), and pathological conditions through either classical, alternative, or lectine pathways leading to the formation and transient insertion of C5b-9/Mac pore complex into cellular plasma membrane. We hypothesise that MAC-insertion promotes a sudden, significant and transient water and Ca^{2+} influx, leading to: (i) endocytosis of the affected area, followed by delivery of C5b-9/MAC-containing plasma membrane into the multi vesicular body (MVB), and its incorporation into exosomes, or (ii) exocytosis of the C9 channle/MAC-affected plasma membrane patch followed by microvesicles (MVs) formation. In addition, the size of the MAC/C5b-9 pore, 12 nm, is large enough to: (i) allow cytoplasmic RNA species to be transferred into the MVB following endocytosis of C5b-9/MAC-containing plasma membrane, and (ii) RNA species located near the plasma membrane to be released in the extracellular space upon C5b-9/MAC insertion.
Methods: Freshly isolated human red blood cells or HUVEC cells were incubated with low concentrations of purified complement components C5-C9 for 20 minutes in the presence of calcium and magnesium. The EV and EV-free fractions were collected and analysed for protein and RNA composition, and the presence of C9 channel in the EV fraction and cellular localisation and organelle distribution of C5b-9 in HUVEC cells analysed by fluorescence and electron microscopy.
Results: Our results showed that when purified human *red blood cells* (RBCs) undergo sub-lytic complement activation (no haemoglobin release), there is an increase in the numbers extracellular RBC-derived vesicles, as well as the in concentration RBC-derived exRNAs, especially miR451, miR92a, and miR7b in the supernatant. The exRNAs species are found both in the EV as well as in the EV-free factions. Proteomic analysis of RBC-derived EVs identified, in addition to MAC/5b-9 pore complex, increased amounts of GPI-anchored complement regulatory proteins, CD55 and CD59, confirming our previous data showing that the insertion of MAC/C5b-9 channel takes place in cholesterol-rich domains. Co-localisation studies using *vascular endothelial cells* and molecular beacons, place MAC/C5b-9, and specific miRNAs into the MVB, suggesting a possible role for MAC/C5b-9 in miRNAs loading into exosome. Moreover, time-lapse qPCR experiments using cell supernatants also indicated a gradual "unloading" of exRNAs from the EV-into the EV-free faction, suggesting that the extracellular vesicles could "leak" through C5b-9/MAC-pore, long after EVs are released from the parent cells, thus explaining several new and unexpected published findings describing high concentrations of blood exRNAs outside of EV fractions.
Conclusion: Our results, for the first time implicate MAC/C5b-9 as: (i) a channel responsible for exosomes and microparticle biogenesis, and (ii) loading of cytosolic RNAs into the exosomes, and (iii) the direct release of cytoplasmic RNA species into the circulation (exRNAs).

OT4.02

Physical coherence and network analysis reveals NEDD4 as novel regulator of exosomal biogenesis

Sushma Anand[1], David Chisanga[2], Shivakumar Keerthikumar[3], Natalie J Foot[4], Sharad Kumar[4] and Suresh Mathivanan[3]

[1]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Australia; [2]La Trobe University, Melbourne, Australia; [3]La Trobe Institute for Molecular University, Melbourne, Australia; [4]Centre for Cancer Biology, University of South Australia, Adelaide, Australia

Introduction: Exosomes are small membrane extracellular vesicles that are secreted under physiological and pathological conditions. However, very little is known about the biogenesis of exosomes. Here, we studied ESCRT dependent mechanism of exosomal biogenesis using physical coherence and network based analysis.
Methods: Using a conserved interaction network and physical coherence model, we indented to identify novel regulators of exosome secretion. To remove literature bias on the protein-protein interaction models, we performed physical interaction enrichment analysis and identified ESCRT protein neighbours that could regulate exosomal biogenesis. Next, we evaluated the role of novel regulators, NEDD4 and STAMBP in exosomal secretion by molecular biology and biochemical experiments.
Results: A total of more than 50 proteins including NEDD4, SDCBP and STAMBP with significant p-values were identified. SDCBP has already been implicated in exosomal biogenesis in previous studies and hence validates our approach. In this study, we investigated the role of the E3 ligase NEDD4 in the biogenesis of exosomes. To address this, we generated CRISPR based NEDD4 knockout (KO) in LIM1215 colorectal cancer cells. In addition, we utilised MEFs from Nedd4 KO mice. Exosome were isolated from wild type (WT) and KO cells using differential centrifugation coupled with ultracentrifugation. The isolated exosomes were quantified based on nanoparticle tracking analysis (NTA) and total protein amount. The analysis revealed significant reduction in exosome secretion in NEDD4 KO cells compared to WT cells suggesting that NEDD4 is a novel regulator of exosomal biogenesis. Furthermore, immunoblotting was performed to confirm the reduced levels of exosomal enriched markers such as Alix and TSG101. Follow-up quantitative proteomic analysis of exosomes derived from WT/KO cells revealed protein cargo dependent on NEDD4. Hence, the results validate the predictions based on physical coherence and network models.
Summary: Overall, in this study we have identified novel regulators of exosomal biogenesis using an integrated bioinformatics and experimental approach.

OT4.03

The EBV LMP1 interactome contains ESCRT-dependent and independent extracellular vesicle sorting proteins

Mark A. Rider, Mujeeb Cheerathodi, Stephanie N. Hurwitz, Lauren A. Howell, Xia Liu and David G. Meckes

Florida State University College of Medicine, FL, USA

Introduction: The Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) is an oncogene required for immortalising resting B lymphocytes and also plays a key role in the transformation of non-lymphoid tissue. The discovery and characterisation of LMP1 protein-protein interactions will likely generate new targets to treat EBV-associated cancers. Unfortunately, classical molecular tools for

identifying protein associations are restrictive. Immunoaffinity purification techniques, for example, rely on harvesting stable protein complexes that are frequently difficult to isolate, and often overlook proteins with transient or weak interactions.
Methods: In this study, we define the broader LMP1 interactome using the recently developed BioID method. We fused the bacterial biotin protein ligase (BirA) to LMP1 and harvested biotinylated target proteins; the biotin 'tag' indicated proteins with vicinal, transient, or stable associations with LMP1.
Results: Using mass spectrometry, we identified over 1000 proteins across seven independent experiments with direct or indirect relationships to LMP1. Additional Significance Analysis of INTeractome (SAINT) analysis assigned confidence scores to potential LMP1 protein-protein interactions. Over 400 proteins had a high SAINT score of greater than or equal to 0.8. Pathway analysis suggested that a significant number of the proteins identified are involved in signal transduction and endosome trafficking. Interestingly, a large number of proteins thought to be important in exosome formation and protein targeting were recognised as probable LMP1 interacting partners, including CD63, syntenin-1, ALIX, TSG101, Hrs, CHMPs, and sorting nexins.
Summary: It is likely that LMP1, which signals from endosomal membranes and is secreted from cells in exosomes, modifies protein trafficking throughout the cell. By way of manipulating the endosomal pathways, LMP1 may exert its oncogenic effects on the surrounding microenvironment.

Room: Metropolitan Ballroom East
Symposium Session 5 – EVs in Tumour Biology
Chairs: Michael Freeman and Carolina Soekmadji 1:30–2:15 p.m.

OT5.01

NAPG can regulate tumour-specific EV secretion

Yusuke Yoshioka[1], Nobuyoshi Kosaka[2] and Takahiro Ochiya[1]

[1]Division of Molecular and Cellular Medicine, National Cancer Centre Research Institute, Japan; [2]National Cancer Centre Research Institute

Introduction: Extracellular vesicle (EV) transfer of cancer pathogenic components enables long-distance crosstalk between cancer cells and distant organs, resulting in the promotion of the initial steps for pre-metastatic niche formation. Therefore, understanding the critical molecular mechanisms underlying the secretion of EVs in cancer cells is an important issue for developing novel therapeutic strategies. The aim of this study is to identify EV secretion-suppressive miRNAs (ESS-miRNAs) and its target genes.
Methods: We used an original screening system based on ExoScreen assay for monitoring CD9 or CD63 positive EV secretion (1). In this assay system, EVs are captured by two types of antibodies, which are detected by photosensitiser beads. One is a biotinylated antibody and the other is an antibody conjugated to AlphaLISA acceptor beads. Using this screening system and a miRNA mimic library containing 2042 miRNAs, ESS-miRNAs were identified in the breast cancer cell line MDA-MB-231D3H2LN, and the prostate cancer cell line PC-3ML. The particle number of EVs was determined using a NanoSight.
Results: Based on the screening result, 4 miRNAs were selected as putative ESS-miRNAs. These miRNAs were further validated by ExoScreen and NanoSight. As a result of the validation, miR-194 was found to inhibit EV secretion in cancer cells. Moreover, NSF attachment protein gamma (NAPG) which appear to be a general component of the intracellular membrane fusion apparatus, was identified as a target gene for miR-194. The knockdown of NAPG also inhibited EV secretion in cancer cells. The suppression of NAPG decreased lung dissemination of breast cancer cells in an orthotopic mouse model.
Conclusion: Here we identify EV secretion-suppressive miRNA and its target gene, and find that the knockdown of NAPG suppresses lung metastasis. These results pave the way towards identifying new therapeutic targets for preventing metastatic spread.

Reference

1. Yoshioka Y., et al., *Nat. Commun.*, 2014.

OT5.02

Intercellular communication between melanoma and stroma cells induce PD-1 overexpression and tumour progression

Edina Gyukity-Sebestyen[1], Mária Harmati[1], Gabriella Dobra[1], Istan B. Nemeth[2], Johanna Mihály[3], Agnes Zvara[1], Eva Hunyadi Gulyas[1], Robert Katona[1], Istvan Nagy[1], Peter Horvath[1], Tibor Pankotai[4], Miklós Erdélyi[4], Zoltan Janos Vereb[5], Tamas Biro[3], Lajos Kemeny[2] and Krisztina Buzas[1]

[1]Biological Research Centre, Hungarian Academy of Sciences, Budapest, Hungary; [2]Department of Dermatology and Allergology, University of Szeged, Szeged, Hungary; [3]Departments of Immunology and Physiology, University of Debrecen, Debrecen, Hungary; [4]Faculty of Science and Informatics, University of Szeged, Szeged, Hungary; [5]Department of Ophthalmology, University of Szeged, Szeged, Hungary

Introduction: PD-1 is a member of the T cell regulator membrane protein family and one of the most promising therapeutic targets in metastatic melanoma. It has been described that highly invasive melanoma subpopulation overexpress PD-1 and the increased PD-1:PD-L1 interaction can promote tumour growth via the mTOR pathway. However, the background of the elevated PD-1 production is not known. To clarify this, we investigated the intercellular exosomal communication between cancer cells and tumour matrix.
Methods: *In vitro*, we modelled the tumour stroma with adipose-derived mesenchymal stem cells (MSCs) and investigated their interaction with melanoma exosomes. *In vivo*, the classical B16F1-C57BL/6 mice model was used. To follow PD-1 expression, Western blot, immunocytochemistry and STORM were used. To describe changes in oncogenes and tumour suppressor genes, we used a customised Life Technologies qPCR panel with 44 genes. The potential interactions between genes were analysed by ingenuity pathway analysis.
Results: We demonstrated that melanoma exosomes upregulate PD-1 and induce a genetic reprogramming in MSCs *in vitro*. The qPCR panel showed clear oncogenic dominance in exosome-exposed MSCs. These cells showed delayed apoptotic response and started to express melanoma specific markers, such as MLANA and MITF. In our *in vivo* model, tumour bearing mice injected with re-educated MSCs i.v. suffered from very fast progression of metastatic disease and the oncogenic dominance of gene expression profile was detected in the lung of the animals also.
Conclusion: These results suggest that melanoma exosomes re-educate MSCs, which show a skewed balance towards a melanoma stem cell-like phenotype. Elevated PD-1 expression and melanoma specific markers also indicate a cancerous transformation of stem cells. Taken together, communication by cancer exosomes enhances the cancerous microenvironment via re-education of stem cells in the tumour matrix.
Funding: This research was funded by OTKA K 112493, GINOP-2.3.2-15-2016-00001.

OT5.03

Zebrafish: a new animal model to study tumour EVs *in vivo*

Vincent Hyenne[1], Shima Ghoroghi[2], Jack Bauer[2], François Delalande[3], Christine Carapito[3], Mayeul Collot[4], Andrey Klymchenko[4], Sebastien Harlepp[5], Lefebvre Olivier[2] and Jacky G. Goetz[2]

[1]INSERM U1109 /CNRS; [2]INSERM U1109; [3]IPHC UMR7178 CNRS/Unistra; [4]UMR7213 CNRS; [5]IPCMS/INSERM U1109

Tumour extracellular vesicles (EVs) are key mediators of the intercellular communication between tumour and stromal cells. This communication can occur locally or at distance and fosters metastatic progression. However, local or distant dissemination of tumour EVs has only been poorly characterised in living organisms. In particular, how EVs circulate in the blood flow, how they cross the endothelial barrier or how specifically they are uptaken by stromal cells is not known. EVs are hundreds of nanometres sized objects and are thus difficult to track *in vivo*. Moreover, adapted model organisms are lacking. We decided to use exploit the multiple advantages of the zebrafish (ZF) embryo to study tumour EVs *in vivo*. The ZF embryo is perfectly suited for intravital imaging with high spatial and temporal resolution and recently emerged as a valid model in cancer biology. We labelled EVs purified from different cancer cell types using our newly developed and highly specific lipid binding Membright dye. Upon injection in the blood circulation, we successfully tracked individual flowing EVs using high-speed confocal imaging. We could determine their average speed in the blood flow, their dependence on hemodynamic profiles as well as their preferential sites of arrest in the vasculature. Furthermore, we identified the main cell types targeted by the injected EVs: endothelial cells and macrophages. Using a correlated light and electron microscopy approach, we described the compartments storing the uptaken EVs. Besides, we demonstrated that ZF melanoma cells secrete EVs containing some recognised exosomal markers, as identified by mass spectrometry. Using these cells, we generated tumours in the ZF embryos and developed tools to follow EVs naturally released by these tumour cells. Therefore, our works establishes the ZF embryo as new model to study tumour EVs *in vivo* and will allow to tackle essential aspects of the biology of tumour EVs in the future.

Room: Harbour Ballroom
Symposium Session 6 – EVs in Inflammatory Diseases
Chairs: Edit Buzas and Rienk Nieuwland 1:30–2:15 p.m.

OT6.01

Annexin-A5 is targeted by heme during haemolysis and fails to block externalised phosphatidylserine in extracellular vesicles during Sickle cell disease

Sihem Sadoudi[1], Sylvain Le Jeune[2], Dominique Charue[1], Laurent Kiger[3], Lubka Roumenina[4], Chantal M Boulanger[1] and Olivier P. Blanc-Brude[1]

[1]INSERM UMR970 – Paris Cardiovascular Research Centre (ParCC); [2]Assistance Publique-Hôpitaux de Paris, Hôpital Avicenne, Sickle Cell Disease Expertise Centre; [3]INSERM UMR955, Etablissement Francais du Sang, IMRB, Hôpital Henri Mondor; [4]INSERM UMR1138, Centre de recherche des Cordeliers

Intravascular haemolysis, such as in sickle cell disease (SCD), is characterised by red blood cell damage, high levels of cell-free heme and extracellular vesicles in plasma, along with inflammation and tissue injury. Stressed leukocytes, platelets, endothelial and red blood cells shed microparticles (MP) that bear externalised phosphatidylserine (PS) at their surface and promote tissue injury. Conversely, intracellular annexin-A5 acts as an inhibitor of externalised PS at the surface of cells and MP. Annexin-A5 is thought to orchestrate vesicle trafficking, promote cell membrane repair, protect against PS-mediated effects and enforce anti-inflammatory and anti-thrombotic control. We investigated a possible functional relationship between intravascular haemolysis and annexins. We hypothesised that annexin-A5 activity is blocked by extracellular heme as it comes into contact with plasma during intravascular haemolysis. In order to test this, we measured PS-, PS+, CD235a+ and annexin-A5+ MP in adult SCD patient and matched control plasmas. We explored annexin-A5 expression in plasma and blood cells by Western blots and ELISA, and also quantified the PS-binding functionality of plasma annexin-A5. Immunocapture of plasma annexin-A5 revealed a strong association with heme (Abs398 nm signature) during SCD, especially during acute haemolytic events. In SCD plasma, we found increased total annexin-A5, but virtually undetectable levels of functional annexin-A5, contrary to controls. This implied a greatly reduced ratio of functional annexin-A5/circulating PS+ MP. Moreover, purified heme bound to annexin-A5 with relatively high affinity *in vitro*, as demonstrated using absorbance shift, autofluorescence quenching and plasmon surface resonance assays, with human serum albumin and hemopexin in competition tests. Haemoglobin and heme also triggered annexin-A5 aggregation *in vitro*, producing high molecular weight and heat-resistant multimers, observed by western blot. Finally, heme completely prevented the binding of exogenous annexin-A5 to plasma and purified PS+ MP, as well as their subsequent detection by flow cytometry. Together, our data suggest that PS-neutralising annexin-A5 is inhibited by cell-free heme, contributing to the accumulation of PS+ MP in plasma during intravascular haemolysis.

OT6.02

Impact of ageing on plasma extracellular vesicle concentration, protein profile and internalisation by leukocytes

Nicole Noren Hooten[1], Erez Eitan[1], Jamal Green[1], Monica Bodogai[1], Nicolle Mode[1], Rikke Baek[2], Malene M Jorgensen[2], Kenneth Witwer[3], Alan Zonderman[1], Arya Biragyn[1], Mark Mattson[1] and Michele Evans[1]

[1]National Institute on Aging, National Institutes of Health; [2]Department of Clinical Immunology, Aalborg University Hospital, Aalborg, Denmark; [3]The Johns Hopkins University School of Medicine, MD, USA

Introduction: Extracellular vesicles (EVs), including exosomes, microvesicles and apoptotic bodies, are released by cells into the circulation. EVs mediate intercellular communication between both neighbouring and distant cells and have biological and physiological roles in both homeostatic and pathological conditions. Emerging roles for EVs in age-related diseases, including cancer, neurodegenerative, metabolic, and cardiovascular disease, suggest that EVs have the potential to be useful for diagnostics as well as for therapeutics. However, the majority of research thus far has focused on identifying differences in EVs when comparing disease states and matched controls.
Methods: We wanted to examine age-related changes in circulating plasma EVs. We isolated plasma EVs from a sub-cohort of the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study, which is a longitudinal, epidemiologic study of ageing. This sub-cohort consisted of young, middle-aged and old individuals (n = 74), who had contributed plasma at two different time points to allow both cross-sectional and longitudinal analyses.
Results: Importantly, we found that EV concentration decreases significantly with human age both in our cross-sectional and longitudinal analyses. We also report that lifestyle factors including body-mass index and smoking also affect EV concentration. To examine whether decreased concentration with age is due to an increase in internalisation by circulating cells, we established a FACS-based assay to measure the internalisation of EVs by PBMCs. EVs from older individuals /were more readily internalised by B cells and increased the expression of the activation marker MHC-II on monocytes compared with EVs from younger individuals, indicating that the decreased concentration of EVs with age may be due in part to increased internalisation. In addition, we identified EV proteins that were significantly changed with age. Interestingly, we also report a significant similarity of both EV concentration and protein amount in individuals over time.
Conclusions: This study provides important insight into establishing an EV profile with human age, which will further aid in the development of EV-based technologies for diagnostics and therapies for ageing and age-related diseases.

OT6.03

Age-related changes in miRNA expression profiles in extracellular vesicles in the murine post traumatic OA model

Ok Hee Jeon[1], David Wilson[2], Bonita Powell[3], Jordan Green[2], Kenneth Witwer[3] and Jennifer Elisseeff[2]

[1]Johns Hopkins University, MD, USA; [2]Johns Hopkins University, Department of Biomedical Engineering, MD, USA; [3]The Johns Hopkins University School of Medicine, MD, USA

Introduction: Ageing and trauma are risk factors for the development of osteoarthritis (OA), a degenerative joint disease with accompanying cartilage degradation, persistent pain and impairment of mobility. However, the underlying mechanisms remain unclear, impeding the development of therapeutic interventions that might prevent or treat the disease. Recently, it has been reported that extracellular vesicles (EVs) play a role in ageing. The expression of their miRNAs changes during ageing and may alter the environment of joint tissue by modulating extracellular matrix degradation and inflammation. In this study, our objective is to examine EV-derived miRNA expression in synovial fluid, where main place for the joint inflammation, from young and old mice using a post-traumatic OA model created by anterior cruciate ligament transection (ACLT).
Methods: We performed the ACLT surgery on mice aged 10 weeks and 19 months and collected the synovial fluid by injecting and aspirating saline on 4 weeks post surgery. EVs were purified by differential ultracentrifugation of the synovial fluid and the morphology and size of EVs were characterised with transmission electron microscopy and nanoparticle tracking analysis. RNA was isolated using Exiqon biofluids kits and miR-34, -146a and -128a were quantified by real-time quantitative PCR before performing the profiling assays to measure all miRNAs simultaneously.

Results: miR-34, which is known to be downregulated cartilage-related extracellular matrix synthesis and upregulated during ageing, -146a and -128a, which is known to be controller of inflammation and reactive oxygen species production in the joint, were enhanced in the aged mice with ACLT surgery compared to young mice with and without surgery and aged mice without the surgery as well.

Conclusion: EV from synovial fluid-derived miRNAs contribute to the development of post-traumatic OA during ageing by transferring miRNA-34, -146a and -128a. These results suggest that EV-derived miRNAs may be biomarker of ageing and could be potential therapeutic targets for treating trauma and age-related degenerative joint disease.

Room: Metropolitan Ballroom West and Centre
Oral with Poster Session 1
Chair: Thomas Kislinger 2:15–3:00 p.m.

OPT01.01 = PT01.04

Amoeboid cancer cells shed extracellular vesicles enriched with nuclear derived material

Mariana Reis Sobreiro[1], Jie-Fu Chen[1], Samantha Morley[1], Sungyong You[1], Kenneth Steadman[1], Navjot Kaur Gill[2], Gina C-Y Chu[1], Leland W.K. Chung[1], Hisashi Tanaka[1], Wei Yang[1], Amy C. Rowat[2], Hsian-Rong Tseng[2], Edwin M. Posadas[1], Dolores Di Vizio[1] and Michael R. Freeman[1]

[1]Cedars Sinai Medical Center; [2]University of California, CA, USA

Introduction: Deformation of the nucleus is required for migrating cells to pass through interstitial tissue spaces. However, it remains unexplored how cells modify nuclear stiffness during metastasis. Cancer cells exhibiting an "amoeboid" phenotype migrate in a manner that resembles neutrophil movement, in which nuclear deformation plays a critical role. Amoeboid tumour cells are characterised by their plasticity, ability to rapidly move through extracellular matrixes and high rates of shedding of extracellular vesicles (EVs).
Methods: Mass spectrometry, flow cytometry, differential centrifugation, iodixanol gradient, confocal 3D imaging, time lapse video microscopy, western blot, NanoVelcro Chip.
Results: Here we demonstrate that stable disruption of nuclear structure by silencing DIAPH3, emerin, or lamin A/C promotes conversion to the highly metastatic amoeboid phenotype in prostate and breast cancer cells. These amoeboid cells produced vesicles from nuclear blebs, underwent shedding of non-apoptotic EVs containing DNA, and exhibited increased sensitivity to inhibitors of DNA damage repair. Amoeboid features were detected in high grade prostate cancer, and capture of circulating tumour cells in mice and patients with metastatic prostate cancer.
Conclusion: These findings suggest the potential of incorporating the use of biomarkers of amoeboid tumour cells into clinical strategies for precision medicine.

OPT01.02 = PF04.01

Extracellular vesicles derived from cancer-associated fibroblasts may have a role in oral cancer invasion

Mauricio R. Dourado[1], Johanna Korvala[2], Raija Sormunen[3], Ilkka Miinalainen[4], Sami Yokoo[5], Pirjo Åström[2], Adriana Franco Paes Leme[5], RIcardo Della Coletta[1] and Tuula Salo[6]

[1]Department of Oral Diagnosis, Piracicaba Dental School, Unicamp, Piracicaba, Brazil; [2]Cancer and Translational Medicine Research Centre, University of Oulu, Oulu, Finland; [3]Biocenter Oulu, University of Oulu, Oulu, Finland; [4]Biocenter Microscopy Service, University of Oulu, Oulu, Finland; [5]Mass Spectrometry Facility, LNBio-CNPEM; [6]Medical Research Centre, University of Oulu, Oulu, Finland

Introduction: A major constituent of the tumour microenvironment are the cancer-associated fibroblasts (CAFs), known to participate in tumour initiation and progression. The communication between stroma cells and tumour is essential for cancer progression, therefore we aim to study extracellular vesicles (EVs) as mediators for the interplay between CAFs and tumour cells.
Methods: Following the ethical guidelines, five CAF and five oral normal fibroblasts (ONF) cell lines were isolated from tumour surrounding and healthy tissue, respectively, and tested for CAF markers (e.g. α-SMA) using qPCR. EVs were isolated using differential ultracentrifugation, and DC protein assay (BioRad®) was used for protein quantification. EV characterisation was accessed by nanoparticle tracking analysis, immunoelectron microscopy (IEM) and Exo-check antibody array (System Biosciences®). Scratch wound healing assay was used to access migration of cancer cells and the invasion assays were carried out using an organotypic model based in leyomioma tissue. Proliferation (BrdU®), viability (MTT®) and apoptosis (FACScan™) of treated cells were also evaluated. To access the EVs proteomic cargo we used mass spectometry (Orbitrap, Thermo Scientific™) and cDNA microarray (Affymetrix®) to analyse the changes at RNA level in HSC3 cells treated with EVs from CAF/ONF.
Results: Nanosight NS300 showed most of the vesicles between 100–200 nm and variable concentration. CAFs EVs had stronger signal against Flotilin, CD81 and Alix in the antibody array when compared to ONF EVs. CAFs EVs increased HSC3 and SAS cells invasion and HSC3 migration. In myoma tissue, CAFs EVs induced deeper invasion with increased spreading of HSC3 cells. Proliferation was not affected by EVs treatment, but the viability decreased and apoptosis rate increased. Using bioinformatic tools, proteomic and cDNA microarray data were combined for possible targets selection.
Conclusion: CAF EVs treatment increased cancer invasion, tumour spreading and migration of aggressive oral cancer cell lines. It also seems to reduce cell viability and induce apoptosis. The selected targets will help to elucidate the mechanisms behind that phenotype.

OPT01.03 = PF03.01

Identification of non-invasive prostate cancer biomarkers by miRNA deep sequencing analysis of urinary extracellular vesicles

Marta Rodriguez-Moreno[1], Cristina Bajo-Santos[2], Viktor Berge[3], Aija Linē[4] and Alicia Llorente[1]

[1]Oslo University Hospital-The Norwegian Radium Hospital, Oslo, Norway; [2]Latvian Biomedical Research and Study Centre /University of Latvia, Riga, Latvia; [3]Department of Urology, Oslo University Hospital, Oslo, Norway; [4]Latvian Biomedical Research and Study Centre, Riga, Latvia

Introduction: Prostate cancer (PCa) is the most commonly diagnosed male malignancy and the second leading cause of cancer-related death in males in the Western world. Suspected PCa patients usually undergo prostate biopsy, but this is an invasive procedure that may over- or underestimate the grade or extent of pathology. To improve the diagnosis of PCa novel biomarkers are therefore needed. Cancer-derived extracellular vesicles (EVs) contain specific mRNAs and microRNAs (miRNAs) that can be analysed in biological fluids in a non-invasive way, and these molecules are being investigated as potential cancer biomarkers with diagnostic and prognostic potential. The aim of this study was to identify miRNAs in urinay EVs that can distinguish prostate cancer patients from healthy donors.
Methods: For this purpose, urinary EVs from 30 individuals (10 healthy controls and 20 PCa patients at different stages of the disease) were isolated by sequential centrifugation. Then, RNA was isolated from the samples and the miRNAs cargo was determined by next generation sequencing.
Results: Comparative analysis of the approx. 254 microRNAs identified in EVs isolated from cancer patients and controls revealed 7 miRNAs differentially downregulated in exosomes of PCa patients. Interestingly, several of these small RNAs have previously been associated with PCa in tissue samples.
Conclusion: Our data suggest that detection of specific EVs miRNAs in urine samples may serve as a liquid biopsy enabling patient stratification and monitoring of treatment response. The expression of the deregulated miRNAs is currently being validated by qRT-PCR in an independent cohort.

OPT01.04 = PF06.08

Pancreatic cancer ExoNet

Carolina de Freitas Ruivo[1], Tiago Gama[2], Carlos Melo[3], José Machado[4] and Sónia Melo[4]

[1]i3S – Instituto de Investigação e Inovação em Saúde; [2]i3S; [3]The Gurdon Institute, University of Cambridge, Cambridge, United Kingdom; [4]i3S – Ipatimup

Intra-tumour heterogeneity represents a major challenge for cancer treatment. The different clones and cancer cell subpopulations in a tumour present distinct tumorigenic capabilities and therapy resistance. Intercellular communication is a key mechanism used by cancer cells to communicate amongst them, with microenviroment and other cells of the body. Communication between subpopulations of cancer cells supports their cooperation to drive tumour progression and to potentiate tumour's response to therapy. Exosomes are extracellular vesicles which play a central part in cell–cell communication. Exosomes are capable of horizontal reprogramming and re-education through the delivery of their cargo to recipient cells.

We have identified five subpopulations of pancreatic cancer cells based on cell surface markers (EpCAM, CD24, CD44 and CD133) which discriminate cells with different tumorigenic and self-renewal capacity. Using stable clones of cancer cells that express exosomes markers fused with fluorescent reporter proteins and secrete colour-coded exosomes, we have studied the flow of exosomes between distinct subpopulations of cancer cells in co-culture. The flow of exosomes was studied in the absence and presence of a standard care chemotherapy agent used for pancreatic cancer, and evaluated by confocal microscopy and flow cytometry.

Here we show that subpopulations of cancer cells communicate with each other via exosomes through an organised dynamic communication network (ExoNet). The ExoNet reshapes in the presence of therapy to allow the tumour to respond and overcome the challenge. The presence of multicolor positive cells showed that exosomes are exchanged between different cancer cell subpopulations forming distinct routes of communication. The flow of exosomes is not a random process and occurs more frequently between specific subpopulations of cancer cells forming an organised network, which supports tumour growth. The established ExoNet is dynamic and reshapes in the presence of gemcitabine and cancer associated fibroblasts. Therefore, we have demonstrated that subpopulations of cancer cells communicate between them in an organised way using exosomes and form a dynamic network of communication, which conveys the tumour with plastic properties that allows it to adapt in face of therapy.

OPT01.05 = PS02.02

Enzymatic exosomes with GPI-anchored hyaluronidase for enhanced tumour penetration and anti-tumour efficacy

Yeon-Sun Hong[1], Yoosoo Yang[2] and In-San Kim[2]

[1]KU-KIST Graduate School of Converging Science and Technology, Seoul, Republic of Korea, Korea University, Seoul, Republic of Korea; [2]Korea Institute of Science and Technology, Seoul, Republic of Korea

Given the physiological abnormalities in tumours, multiple biological barriers need to be overcome before nanomedicines are delivered to the target site. Here we report an exosome-based strategy that overcomes the immunosuppressive and anti-cancer therapy resistant tumour microenvironment with overly accumulated extracellular matrix. This enzymatic exosome harbouring native PH20 hyaluronidase (Exo-PH20) could penetrate deeply into tumour foci *via* hyaluronan degradation, allowing tumour growth inhibition and increased T cell infiltration into tumour. In addition, exosome-mediated simultaneous delivery of PH20 hyaluronidase and chemotherapeutics (Doxorubicin) triggers synergistic effect on the tumour growth inhibition with a low dose of drug. This exosome is designed to degrade hyaluronan on its moving paths, thereby augmenting nanoparticle penetration and drug diffusion. Note that, engineered exosome with native GPI anchored form of hyaluronidase has higher enzymatic activity than truncated form of recombinant protein. Our results provide the promising exosome-based platform harbouring membrane-associated enzyme with increased activity. We expect that the enzymatic exosome has potential for use as a biologically active drug delivery vehicle in treating cancers.

OPT01.06 = LBO.01

Mesenchymal stem cell derived exosomes mediate neurovascular protection

Johnathon D. Anderson, Jan Nolta, Peter Belafsky, Maggie Kuhn and Greg Farwell

University of California Davis Medical Center, CA, USA

Introduction: Mesenchymal stem cells (MSCs) facilitate functional recovery in numerous animal models of inflammatory and ischemic tissue related diseases with a growing body of research suggesting that exosomes mediate many of these therapeutic effects. However, it remains unclear which types of proteins are packaged into exosomes as compared to the cells from which they are derived.

Methods: Using high-resolution isoelectric focusing coupled liquid chromatography tandem mass spectrometry, we have previously reported that MSC derived exosomes are packaged with angiogenic proteins and functionally induce angiogenesis under ischemic conditions. Here, using comprehensive proteomic analysis, we demonstrated that exosomes are packaged with a markedly higher fraction of specific protein subclasses as compared to their cells of origin, indicating regulation of their contents. We also demonstrated the therapeutics effects of MSC exosomes in two animal models, ischemic stroke and diabetic retinopathy. We also characterized the metabolomic and lipidomic composition of MSC exosomes using mass spectrometry.

Results: We find that MSC exosomes are packaged with distinct classes of proteins, metabolites and lipid membrane components. We demonstrate that MSC exosomes improve outcomes in two models of ischemic tissue diseases, ischemic stroke and diabetic retinopathy.

Summary/Conclusion: MSC exosomes hold the potential to be used as a novel therapeutic platform which holds several advantages over the use of MSCs.

Room: Metropolitan Ballroom East
Oral with Poster Session 2
Chair: Uta Erdbruegger 2:15–3:00 p.m.

OPT02.01 = PT03.01

Protective role of extracellular vesicles in diabetic microangiopathy

Chiara Gai, Tatiana Lopatina, Yonathan Gomez, Maria Felice Brizzi and Giovanni Camussi

Department of Medical Science, University of Turin, Torino, Italy

Introduction: Diabetic microangiopathy is a pathological process ending in endothelial dysfunction and vascular lesions. Adipose mesenchymal stem cells (ASCs) are a population of multipotent adult stem cells with immunosuppressive, anti-inflammatory, and regenerative properties. It has been previously described that extracellular vesicles (EVs) derived from ASCs (ASC-EVs) possess pro-angiogenic abilities. The aim of the present study was to evaluate whether ASC-EVs may inhibit endothelial cells dysfunction induced by intermittent hyperglycaemia mimicking human microangiopathy condition.
Methods: We set up an *in vitro* intermittent hyperglycemic model by culturing Human Microvascular Endothelial Cells (HMEC) for 7 days with 48 h cycles of high glucose (HG – 25 mM) – normal glucose (NG – 5 mM) exposure. At day 5 HMEC were incubated with a dose of 10×10^3 EV/cell of ASC-EVs or vehicle alone for 48 h. At day 7 we evaluated apoptosis (Annexin V), proliferation (BrdU incorporation), oxidative stress (DNPH), and tube formation ability (Matrigel).
Results: Intermittent high-low glucose (INT HG) induced the onset of a significant decrease of HMEC proliferation, an increased number of apoptotic cells, oxidation of intercellular proteins, and a reduction in the formation of capillary-like structures in Matrigel. Treatment with ASC-EVs significantly restored proliferation, inhibited apoptosis and oxidation, and restored capillary-like formation ability.
Furthermore, to evaluate ASC-EVs mechanism of action, their mRNA cargo was analysed. We observed that ASC-EVs contain high HGF mRNA levels. Therefore, tube formation assay on Matrigel in the presence of ASC-EVs, with or without HGF-receptor inhibitor (crizotinib) was performed. We observed that crizotinib significantly reduced the ASC-EVs-induced capillary-like formation. Microarray analysis of cells treated in different experimental conditions were also performed.
Conclusions: Results of the present study demonstrate that ASC-EVs may inhibit the endothelial dysfunction induced by INT HG, which mimic diabetic microvascular injury. ASC-EVs may, at least in part, exert pro-angiogenic effects by delivering HGF mRNA to recipient endothelial cells and by activating HGF signalling pathway.

OPT02.02 = PT03.02

Significant improvement of survival of rats with acute liver failure by high concentration exosome of human adipose-derived stem cells

Yinpeng Jin, Hongchao Li, Junyi Wang, Lingyu Meng, Li Li, Xiaojin Wang, Chengwei Chen and Qingchun Fu

Shanghai Liver Disease Research Centre, The 85th Hospital of PLA

Introduction: To collect the conditioned medium (CM) of human adipose-derived stem cells (ADSC), obtain exosome through isolation, treat D-gal induced rat model of acute liver failure with ADSC, ADSC exosome and ADSC lysate, respectively, and compare their efficacy and analyse the potential effective elements of ADSC exosome and the underlying mechanisms.
Methods: 1. To obtain ADSC from healthy human abdominal subcutaneous fat tissues through collagenase I digestion and purify the cells through adherent culture, 2. Collect exosome by ultra filtration concentration centrifugation, and evaluate ingredients including proteins and RNAs in the exosome via protein mass spectrometry and gene sequencing, 3. Assess the influence of CM and ADSC co-culture on the proliferation of ADSC through *in vitro* assay, 4. Smash ADSC with ultrasound, and obtain the corresponding cell lysis solution, 5. Construct SD rat model of acute liver failure with biphasic injection of D-gal into the rat abdominal cavity, and treat the acute liver failure rats with ADSC, low concentration lysate solution, high concentration lysate solution, low concentration exosome and high concentration exosome through vena femoralis injection. Observe the survival of the rats, and evaluate the rats and human RNA expression differentiations in the rats' liver tissues in high concentration exosome group and PBS controlled group, 6. Analyse the key genes that function in the treatment procedures of acute liver failure with ADSC exosome by bioinformatics methods.
Results: 1. Highly purified adipose derived stem cells could be obtained through adherent culture. 2. The exosome collected through ultrafiltration concentration centrifugation could be observed and presented as 30–100 nm-size circular goblet membrane vesicle under electron microscope; the protein mass spectrometry identified 1466 kinds of peptides for the exosome and RNA-sequencing identified more than 100 kinds of miRNA for the exosome. 3. In the *in vitro* co-culture experiment, the proliferation rates of ADSC were positively correlated with the concentrations of exosome within a certain concentration ranges. 4. Cell lysis solutions with rich proteins could be obtained by smashing the ADSC through ultrasound. 5. In animal experiment, the survival of the rats in ADSC group, low concentration lysis solution group, high concentration lysis solution group, low concentration exosome group and high concentration exosome group were 37.5%, 25%, 50%, 62.5% and 100%, respectively, whereas in PBS controlled group, the survival of the rats was only 27.3%, therefore, it was speculated that the efficacies of exosome in treating acute liver failure rats were positively correlated with its concentrations. 6. Bioinformatics methods have identified that the lncRNA GADD45AP1 and H19 regulate the phenotype changes of the rat livers in the exosome group through influencing MAPK pathway.
Conclusion: High concentration ADSC exosome has good curative effect for acute liver failure rats, and could improve their survival. lncRNA GADD45AP1and H19 are probably the key genes that function in the treatment procedures for acute liver failure.

OPT02.03 = PT11.01

***In vivo* analysis of the potential of exosomes isolated from menstrual blood-derived mesenchymal stem cells in regeneration of insulin-producing cells in diabetic type 1 animal model**

Elahe Mahdipour

Department of Medical Biotechnology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Introduction: Diabetes type 1 is characterised by the lack of insulin production as a result of degeneration of insulin-producing beta cells in the pancreas. The autoimmune response against beta cells is the main reason for this disease; therefore any strategies that help immune response regulation can be beneficial. Studies have shown the effectiveness of mesenchymal stem cells in regulation of T cell response and pancreatic islet repair. However, application of MSCs accompanies the cell therapy safety issue. The unknown fate of injected stem cells is one of the major safety concerns regarding stem cell therapies; therefore, in this study we have used the exosomal secretome of MSCs to regenerate insulin-producing cells.
Methods: Mesenchymal stem cells were isolated from menstrual blood as a rich and non-invasive source of MSCs. Exosomes were isolated and characterised using western blot and AFM, TEM techniques. Exosomes were injected intravenously at different time points after induction of

diabetes using STZ. Blood glucose and insulin levels were measured at pre-determined time points and animals were sacrificed at day 60 and regeneration of beta cells and insulin production at pancreas were analysed using immunohistochemistry.
Results: Flow cytometric and differentiation assays confirmed the characters of MSCs derived from menstrual blood. The presence of CD81, CD63, Tsg-101, Calnexin markers on exosomes was confirmed using western blotting and AFM and TEM analysis verified the presence of purified exosomes. Altogether, the blood levels of glucose and insulin and the histochemistry analyses represented the regenerative potential of exosomes isolated from menstrual blood-derived mesenchymal stem cells in the restoration of insulin-producing cells.
Conclusion: Although very successful in preclinical studies, mesenchymal stem cells have still very limited therapeutic applications in clinic mainly because of their safety concerns. Secreted exosome from these cells exerts most beneficial properties of stem cells; however, they follow fewer safety issues as they are not active agents as cells are. This work represents the effectiveness of mesenchymal stem cell-derived exosomes in the regeneration of pancreatic beta cells.

OPT02.04 = PF07.01

Stroke extracellular vesicles express inflammatory markers and induce macrophage activation

Yvonne Couch[1], Naveed Akbar[1], Simon Davis[1], Roman Fischer[1], Kim Wals[1], Alex Dickens[2], Ain Neuhaus[1], Annette Burgess[1], Peter Rothwell[1] and Alastair Buchan[1]

[1]University of Oxford, Oxford, UK; [2]University of Turku, Turku, Finland

Extracellular vesicles (EVs) are lipid complexes shed from the membrane of cells, as well as actively exocytosed, as part of normal physiology, but also during pathological processes such as those occurring during a stroke. Here, EVs were quantified and analysed in the sera of patients after an acute stroke (<24 h, Oxford Vascular Study). EV number, but not size, is significantly increased in stroke patients when compared to age-matched controls. The number of EVs does not correlate with NIHSS score, age or levels of circulating TNFr1, but does positively correlate with circulating levels of C-reactive protein and neuron specific enolase. Isolated EV fractions were subjected to untargeted MS/MS followed by principle components and pathway analyses, revealing an overall increase in acute phase response proteins, including active C-reactive protein. Finally, EV fractions were applied to monocyte/macrophage cultures and were found to induce inflammatory gene expression. This data demonstrates for the first time that the EV fraction produced in the acute phase after a stroke is pro-inflammatory in nature and is capable of inducing inflammation in immune cells.

OPT02.05 = PS05.01

Proteomic profiling reveal Src as a novel microvesicle-associated biomarker for myocardial infarction

Olof Gidlöf[1], Mikael Evander[2], Thomas Laurell[1] and David Erlinge[3]

[1]Lund University, Sweden; [2]Department of Biomedical Engineering, Lund University, Sweden; [3]Department of Cardiology, Clinical Sciences, Lund University, Sweden

Introduction: Extracellular vesicles (EVs) are a promising source of plasma biomarkers for a wide array of disease states, including cardiovascular disease. The principal method for isolating EVs from blood is differential centrifugation, but this technique is time consuming and may compromise the integrity of the vesicles. Acoustic seed trapping is a rapid, non-contact alternative to centrifugation for isolation of EVs from plasma. The aim of this study was to compare the proteomic profiles of EVs from patients with myocardial infarction (MI) and healthy controls isolated with acoustic seed trapping or differential centrifugation using a proximity extension based assay to identify novel EV-associated biomarkers for MI.
Method: Plasma EVs were isolated from 10 patients with myocardial infarction and 10 healthy controls, using acoustic seed trapping or differential centrifugation (20,000*g* for 1 h). Protein was extracted from isolated EVs and EV-depleted supernatant and subjected to proteomic profiling using the Proseek Multiplex CVD and Inflammation Panels (Olink Bioscience). EV-associated proteins of interest were confirmed with transmission electron microscopy (TEM), flow cytometry and nanoparticle tracking analysis (NTA).
Results: 90 proteins were detected in EVs or EV-depleted supernatant. 40% of detected proteins were enriched in either of the EV fractions, whereas only 6% were enriched in the EV-depleted supernatant. The protein profile of EVs isolated with acoustic trapping and differential centrifugation was highly similar, as analysed by hierarchical clustering and principal component analysis. 10 proteins were differentially expressed in both EV fractions, and one notable EV-associated protein with significantly higher levels in MI patients was Proto-oncogene tyrosine-kinase Src. The presence of Src on EVs of all sizes (50–800 nm) was confirmed with flow cytometry, TEM and NTA. Src was present on EVs of platelet, endothelial cell and leucocyte origin, as determined by flow cytometry.
Conclusion: There is considerable overlap in the proteomic profiles of EVs isolated with acoustic trapping and differential centrifugation. Src is associated with EVs of different sizes and origins and is a potentially novel biomarker for MI.

OPT02.06 = LBO.02

Cross-talk between monocyte and endothelial cells via Inflammatory Extracellular Vesicles in cardiovascular disease

Baharak Hosseinkhani[1], Nynke Van den Akker[2], Mick Gagliardi[2], Sören Kuypers[1], Daniel G. M. Molin[2] and Luc Michiels[1]

[1]Hasselt University, Biomedical research institute, Martelarenlaan 42, 3500 Hasselt, Belgium; [2]Maastricht University, dept. Of Physiology, Cardiovascular Research Institute Maastricht (CARIM), Universiteitssingel 50, 6200 MD Maastricht, The Netherlands

Introduction: EV-mediated intercellular communication between monocytes (MC) and endothelial cells (EC) plays an active role in vascular inflammation that in turn can lead to cardiovascular diseases. The pro- and anti-inflammatory functional effects of inflammatory EV subpopulations at the site of inflamed vascular cells is poorly understood. Therefore, we aim to unravel the pro/anti-inflammatory responses of MC and EC to inflammatory EV.
Methods: TEM, NTA and western blot were used to study the size distribution and concentration of UC- purified EV from the culture supernatant of HUVEC, either untreated (uEV)or treated with TNF-α to induce an inflammatory stress (tEV). Thereafter, MC and EC in mono and co-culture systems were exposed to the uEV and tEV. Relevant pro/anti-inflammatory markers (IL-1β, IL4, IL-6, IL8, IL10, IL-13, TNF-α, ICAM-1, VCAM-1, PECAM-1, E-Selectin, MCP-1, CD40 and HSP70) were evaluated on RNA level (qPCR) and protein level (ELISA, IF, western blot) in both cell types. the functionality of uEV and tEV were assessed using cell migration and adhesion tests.
Results: EV having an approximate size range between 30-300nm were successfully isolated from EC which can be taken up by MC and EC in culture. We observed that the level of pro-inflammatory markers (IL-1β, IL-6, IL8, ICAM-1, VCAM-1 and MCP-1) in EC and MC treated with tEV at both RNA and protein level were significantly increased while a significant decrease in anti-inflammatory marker (IL4, IL10, IL13 and CD40) was detected. We also discovered that tEV and uEV do induce anti-inflammatory responses in recipient cells as indicated by the increased level of IL4, IL10, IL13 and CD40. Moreover, tEV promoted both the migration of EC and the adhesion of MC to EC.
Summary/Conclusion: Taken together, our current findings confirmed that both pro and /anti-inflammatory cross talk between EC and MC is established via EV-carrying corresponding (RNA and proteins) mediators.
Funding: This work was co-financed by the EU through the Interreg IV Flanders-the Netherlands project VaRiA (IVA-VLANED-3.65) and Interreg V Flanders-the Netherlands project Trans Tech Diagnostics (TTD).

Room: Harbour Ballroom
Oral with Poster Session 3
Chair: Eric Boilard
2:15–3:00 p.m.

OPT03.01 = PT05.05

Cryogenic-temperature electron microscopy imaging of extracellular vesicles shedding

Naama Koifman[1], Idan Biran[1], Anat Aharon[2], Benjamin Brenner[3] and Yeshayahu Talmon[4]

[1]Department of Chemical Engineering and the Russell Berrie Nanotechnology Institute, Technion-Israel Institute of Technology, Haifa, Israel; [2]Department of Hematology, Rambam Health Care Campus; [3]Department of Hematology and Bone Marrow Transplantation, Rambam Health Care Campus; [4]Department of Chemical Engineering and The Russell Berrie Nanotechnology Institute (RBNI), Technion-Israel Institute of Technology

Introduction: Cryogenic scanning electron microscopy (cryo-SEM) is a unique imaging technique, by which cells can be imaged at a high resolution avoiding the addition of fixatives or contrast agents. Cryo-SEM is highly advantageous for imaging shedding cell membranes, which remain unaltered during specimen preparation, thus generating a more accurate and reliable morphological analysis. Moreover, cryogenic temperature electron microscopy is still not widely used for the study of extracellular vesicles (EVs), although it is optimal for the investigation of those systems. The human leukaemia monocytic cell line (THP1) is known to shed EVs under various stimulations. We study the effects of stimulation by exposure to the endotoxin lipopolysaccharide (LPS) or by starvation on THP1.
Methods: Unstimulated and stimulated cells were thermally fixed by high pressure freezing, and imaged by cryo-SEM. EVs isolated from unstimulated and stimulated cells were imaged by cryogenic transmission electron microscopy (cryo-TEM). We also characterised the isolated EVs by nanoparticle tracking analysis (NTA).
Results: Cryo-SEM images show blebbing of cells stimulated by LPS, which is in good agreement with previously suggested models. Micrographs show extensive membrane blebbing as round, vesicular invaginations. Cells that underwent a 48-hour starvation stimulation exhibited a different morphology, including elongated membrane protrusions and shrunken membrane and nucleus. EV morphologies were shown to be highly heterogenous in size and nanostructure. EVs isolated from cells undergoing starvation were fewer and larger than EVs isolated from LPS-stimulated cells.
Conclusions: Cryo-SEM provides a high magnification view of cells undergoing shedding, revealing the size and morphology of the EVs prior to their release from the cell. Cryo-TEM of the isolated EVs complemented by NTA provides a statistical and morphological characterisation of the EVs after their release. Although both starvation and endotoxin-exposure are common stimulations, they most probably lead to a different cellular response, resulting in differences in size and concentration of the isolated EVs.

OPT03.02 = PS04.01

Easy extracellular vesicle detection on a surface-functionalised power-free microchip

Ryo Ishihara[1], Tadaaki Nakajima[2], Asuka Katagiri[1], Yoshitaka Uchino[1], Kazuo Hosokawa[3], Mizuo Maeda[3], Yasuhiro Tomooka[2] and Akihiko Kikuchi[1]

[1]Department of Materials Science and Technology, Tokyo University of Science, Tokyo, Japan; [2]Department of Biological Science and Technology, Tokyo University of Science, Tokyo, Japan; [3]Bioengineering Laboratory, RIKEN

Introduction: Extracellular vesicles (EVs) are expected as novel cancer biomarkers (1). However, rapid and easy EV detection is challenging, hence conventional detection methods require large sample volumes and long detection times. For point-of-care (POC) diagnosis, the microchip-based analytical system has attracted attention because of its cost effectiveness, *i.e.*, small sample sizes with short analysis times. Hosokawa et al. have developed various biomarker detection methods on the portable power-free poly(dimethylsiloxane) (PDMS) microchip (2). Recently, we proposed a surface-functionalised power-free microchip (SF-PF microchip), in which the inner surface of the microchip was chemically modified by radiation beam-induced graft polymerisation (RIGP), as a platform towards POC diagnosis (3). In this study, to detect EVs rapidly and easily, the SF-PF microchip for EV was prepared.
Methods: By UV light-induced graft polymerisation, inner surface of microchannels of the microchip was modified with poly(2-aminoethyl methacrylate). Then, anti-CD63 antibodies were immobilised to the grafted polymer chains. Finally, the obtained microchip was degassed. For EV detection, laminar flow-assisted dendritic amplification (4) was adopted. EVs from mammary epithelial cell line (MCF10A) and breast cancer cell line (MCF7) were isolated by differential ultracentrifugation.
Results: Isolated EVs were characterised by a nanoparticle tracking device (NanoSight). The amount of anti-CD63 antibody immobilised on the surface of the microchannels, which depends on graft initiator and anti-CD63 antibody concentrations, was evaluated by fluorescent-labelled secondary antibody adsorption. The solutions were injected into the microchip by power-free sequential injection. EVs were successfully detected on the SF-PF microchip. The required sample volume was 1.0 μL and the total analysis time was 20 min.
Conclusion: Rapid and easy EV detection on the SF-PF microchip was demonstrated. Since most existing methods take more than a few hours, the proposed microchip-based EV detection method would play an important role in establishment of POC cancer diagnosis.

References

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2. Hosokawa et al., *Lab Chip*, 2004; 4: 181–185.
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OPT03.03 = PS03.01

Sweating the small stuff: extracellular vesicles from sweat

Prateek Singh and Seppo Vainio

University of Oulu, Oulu, Finland

Sweat has been an untouched territory in the extracellular vesicles (EVs) field owing to its complex composition, and lack of standard collection strategies in large volumes. Previously sweat has been used to monitor hydration state, detect drugs of abuse and diagnose cystic fibrosis. We have developed protocol to isolate sweat in a quantifiable manner, and purify EVs from the same. Proteomics has been a powerful tool in identifying and characterise the biochemical composition of exosomes. We present the mass spectrometry data of the sweat extracellular vesicles, providing a valuable bank of potential biomarkers.
Sweat was collected from healthy volunteers performing physical activity sessions. Informed consent was obtained from the volunteers beforehand. The collected sweat was immediately processed for extraction of the extracellular vesicles. Sequential ultracentrifugation was performed to separate cell debris at 1000*g*, apoptotic bodies at 10,000*g* and the extracellular vesicles at 100,000*g*. The vesicles were washed and resuspended in PBS and stored in aliquots at −80 °C. The supernatant from the 100,000*g* spin step was retained.
Transmission and scanning electron microscopy was used to structuraly characterise the vesicles. LC-ESI-MS/MS analyses were

performed on a nanoflow HPLC system coupled to a mass spectrometer equipped with a nano-electrospray ionisation source. MS data was searched against SwissProt database (version 2016_09) with a taxonomy filter "human".
Proteomics analysis yielded 454 proteins identified. The extracellular vesicles contain the characteristic exosome associated proteins, CD63, CD9, Annexin V, HSP90, EGS, and stained positive for CD63 in immunogold electron microscopy.
To the best of our knowledge, we are the first to systematically characterise the extracellular vesicles from human sweat. This study used the most effective method (LC-MS/MS) to identify protein content of sweat vesicles. This will enable rapid diagnostic capabilities using sweat as a source of extracellular vesicles, which are being pursued as putative biomarkers for diseases and health conditions. Sweat has the advantage of being collected non-invasively, like saliva and urine, but unlike them, can be collected from a topical site without the possibility of being adulterated.

OPT03.04 = LBO.03

Monitoring standardised treatment efficacy of multiple sclerosis on molecular level

Fatemeh Vafaee[1], Saeideh Ebrahimkhani[2], Michael Barrnet[3], Catherine Suter[4] and Michael Buckland[3]

[1]Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia, School of Mathematics and Statistics, The University of Sydney, Sydney, NSW, Australia; [2]Brain and Mind Center, Sydney University; [3]Sydney Medical School, Brain and Mind Centre, The University of Sydney, Sydney, NSW 2006 Australia; [4]Victor Chang Cardiac Research Institute

Introduction: Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system. In most MS patients, disease starts with relapsing remitting (RR) symptoms followed by secondary progression. While different effective disease-modifying treatments are currently available, no molecular markers exist to monitor disease progression and treatment efficacy. Additional studies are therefore required to investigate the disease suppression at the molecular level. We aimed to determine the impact of a standardised treatment on small RNAs in serum-derived exosomes.
Methods: We profiled exosomal miRNAs from 33 RRMS patient serum samples in baseline, 6 months and 12 months after starting the treatment along with 21 matched controls using high-throughput sequencing. The RPA Hospital Human Research Ethics Committee ethically approved the study, and all patients provided written informed consent. Full clinical data was accumulated for all patients and healthy individuals.
Results: We reported that RRMS patient sera exhibit dysregulation of miRNAs in relation to the treatment. Furthermore, we used advanced machine learning approaches to identify the predictive power of signatures derived from the discovered miRNAs and characterized dynamic regulatory patterns of miRNAs in active and quiescent phases.
Summary/Conclusion: Circulating exosomes with selective package of small noncoding RNAs represent promising non-invasive, cost effective and accurate detectable biomarker of disease diagnosis and response to therapy. To our knowledge, this is the first proof-of-principle demonstrating that miRNAs from serum exosomes can be used to determine the impact of the standardised treatment to suppress the RRMS disease at the molecular level.

OPT03.05 = LBO.04

Metastatic efficiency is dependent on cell volume loss due to extracellular vesicle release during cancer cell extravasation

Yohan Kim[1], Andrew Chun-Him. Poon[2], Fabrice Lucien[3], Janice Gomes[4], Florence Deng[1] and Hon S. Leong[1]

[1]Western University, Ontario, Canada; [2]University of Western Ontario, Ontario, Canada; [3]Lawson Health Research Institute; [4]University of Western University, Ontario, Canada

Introduction: Metastasis is the main cause of mortality and morbidity in cancer patients. Tumor cells from primary tumor enter the bloodstream (intravasation) and cross the vessel wall (extravasation) to form secondary colonies. Our laboratory has shown previously that inhibiting cancer cell extravasation is a potential target for halting cancer metastasis. Surprisingly, we observe circulating cancer extracellular vesicles (EVs) during cancer cell extravasation *in vivo*. Using intravital imaging, we observe that extravasating cancer cells lose significant cell volume, negatively impacting metastatic colony formation rates. Since induction of necroptosis (programmed necrosis) also resulted in a significant increase of EV release, we hypothesize that inducing cancer cell necroptosis leads to cell volume reduction, inhibition of cell extravasation and metastasis.
Methods: Invasive human breast/prostate cancer cell lines were cultured and injected into the chorioallantoic membrane (CAM) of chicken embryos. We performed intravital imaging of cancer cell EV release and extravasation. To quantitate EVs released from cancer cells, we used a nanoscale flow cytometer to analyze plasmas from the CAMs or conditioned media.
Results: Our results show that an increase in circulating cancer cell EV release significantly reduces extravasation rates of cancer cells and metastatic colony formation rates. Although pro-apoptotic cancer cells released elevated amounts of EVs that resulted in reduced extravasation rates, extravasating cancer cells showed the absence of caspase-3 activity on EV release. Pro-necroptotic cancer cells showed an increase in cancer cell EV release with cell volume reduction and a decrease in cancer cell extravasation rates. Inhibition of intravascular cancer cell necroptosis improved extravasation rates remarkably and reduced EV release drastically.
Summary/Conclusion: Our findings recapitulated that a reduction in cell volume by releasing EVs facilitates extravasation, at the cost of reduced efficiency in forming secondary colonies. Although the pro-apoptotic process of cancer cells can stimulate more EV release, our results on the inhibition of necroptosis and the pro-necroptotic process implicate that necroptosis is an emerging regulator of cancer metastasis.
Funding: Prostate Cancer Canada, Ride for Dad, AMOSO, OGS

OPT03.06 = LBO.05

Exosomal microRNA signatures in multiple sclerosis reflect disease status

Saeideh Ebrahimkhani[1], Fatemeh Vafaee[2], Michael Barrnet[3], Catherine Suter[4] and Michael Buckland[3]

[1]Brain and Mind Center, Sydney University; [2]Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia, School of Mathematics and Statistics, The University of Sydney, Sydney, NSW, Australia; [3]Sydney Medical School, Brain and Mind Centre, The University of Sydney, Sydney, NSW 2006 Australia; [4]Victor Chang Cardiac Research Institute

Introduction: Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). There is currently no single definitive test for MS; current diagnosis and disease monitoring are associated with high cost and have limited utility in distinguishing active inflammatory from progressive disease. Circulating exosomes represent promising candidate biomarkers for a host of human diseases. Exosomes contain RNA, DNA, and proteins, can cross the blood-brain barrier, and are secreted from almost all cell types including cells of the CNS. We hypothesized that serum exosomal miRNAs could present a useful blood-based assay for MS disease detection and monitoring.
Methods: Exosome-associated microRNAs in serum samples from MS patients (n=25) and matched healthy controls (n=11) were profiled using small RNA next generation sequencing.
Results: We identified differentially expressed exosomal miRNAs in both RMS (miR-15b-5p, miR-451a, miR-30b-5p, miR-342- 3p) and progressive MS patient sera (miR-127-3p, miR-370-3p, miR-409-3p, miR-432-5p) in relation to controls. Critically, we identified a group of nine miRNAs (miR-15b-5p, miR-23- 3p, miR-223-3p, miR-374a-5p, miR-30b-5p, miR-433-3p, miR-485-3p, miR-342-3p, miR- 432-5p) that distinguished relapsing-remitting from progressive disease.
Summary/Conclusion: This study shows that serum exosomes from MS patients are meaningfully altered in their miRNA profiles, which can potentially be utilized as biomarkers. To our knowledge, this is the first proof-of-principle demonstration that microRNAs associated with circulating exosomes are informative biomarkers for the diagnosis and monitoring of MS.

Biotech Sponsored Sessions

Room: Metropolitan Ballroom West and Centre

Symposium Session 7: Biotech Sponsored Session 3:30–3:45 p.m.

Room: Metropolitan Ballroom West and Centre
Symposium Session 7 – Emerging Technologies in EV Characterisation
Chairs: Hubert Yin and John Nolan 3:30–5:15 p.m.

OT7.01

Flow cytometric analysis of extracellular vesicle subsets in body fluids: impact of coincidence and swarm by particles of non-interest

Sten F.W.M. Libregts[1], Ger J.A. Arkesteijn[1], Andrea Németh[2], Esther N.M. Nolte-'t-Hoen[1] and Marca H.M. Wauben[1]

[1]Department of Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [2]Department of Genetics, Cell- and Immunobiology, Faculty of Medicine, Semmelweis University, Budapest, Hungary

Introduction: For flow cytometric analysis of extracellular vesicle (EV) subsets in clinical samples, isolation and preparation should be swift and comprise minimal handling, while permitting high throughput, multi-parameter analysis of EVs. One potential approach is to stain EV subsets by directly applying fluorophore-conjugated antibodies to body fluids, after which EVs are isolated and separated from unbound antibody using size-exclusion chromatography (SEC). Here we explored whether fluorescence-based flow cytometric analysis allows reliable detection of subsets of fluorescently labelled EVs against a background of non-fluorescent EVs or differently labelled submicron-sized particles.
Methods: We performed spike-in experiments using various body fluids, PKH67-stained EVs, and various fluorescent and non-fluorescent beads. Where needed, EVs were purified from body fluids using SEC after spiking. Flow cytometric analysis of events of interest was done by performing fluorescence threshold triggering on a BD Influx optimised for the detection of small particles.
Results: We found that upon fluorescence threshold triggering high concentrations of particles of non-interest can severely interfere with the light scatter detection of EVs of interest as a result of coincidence and swarm. When interfering particles were labelled with different fluorophores, the coincidence caused false positivity for these fluorophores on the EVs of interest. We show that by performing serial dilutions and monitoring light scatter and fluorescence parameters, interference of particles of non-interest can be checked and controlled.
Conclusion: Although it is technically possible to detect a subset of fluorescently labelled EVs in a background of non-fluorescent or differently-labelled submicron-sized particles upon fluorescence threshold triggering, our findings imply a precaution for its application on clinical samples in which the ratio between EVs of interest and other particles is unknown and variable.
Funding: This research is supported by the Dutch Technology Foundation STW (Perspectief Program Cancer ID, project 14191), which is part of the Netherlands Organization for Scientific Research (NWO), and which is partly funded by the Ministry of Economic Affairs.

OT7.02

Confounding factors in extracellular vesicle ultrafiltration and protein analysis

Glenn Vergauwen[1], Bert Dhondt[1], Jan Van Deun[1], Evy Timmerman[2], Kris Gevaert[2], Geert Braems[3], Rudy Van den Broecke[3], Veronique Cocquyt[4], Hannelore Denys[4], Olivier De Wever[1] and An Hendrix[1]

[1]Laboratory of Experimental Cancer Research, Cancer Research Institute Ghent (CRIG), Ghent University, Ghent, Belgium; [2]VIB Medical Biotechnology Centre; [3]Department of Gynaecology, Ghent University Hospital; [4]Department of Medical Oncology, Ghent University Hospital, Ghent, Belgium

Introduction: Identification and validation of extracellular vesicle (EV)-associated functions and biomarkers requires robust isolation and characterisation protocols. We assessed the impact of commonly implemented but modified analytical variables on EV analysis.
Methods: We compared five different centrifugal filters that are often used to reduce large volume biofluids or concentrate EVs on three sample types: plasma, urine and EV-spiked PBS. Protein and nanoparticle tracking analysis was performed on the concentrate, membrane and flow through to determine EV recovery. Next, we compared three colorimetric and three fluorometric protein assay kits for their efficiency in measuring protein concentration of EV samples. In all protein assay kits the same sample volume of 5 µL EVs (1×10^{10} particles) was used. The presence and influence of Optiprep™ remnants in EV samples was assessed by DC protein assay kit-based interference of Optiprep™ at 750 nm and Q-Exactive protein analysis respectively.
Results: Regenerated cellulose with 10k pore size generated highest particle and protein recovery of EV-spiked PBS. Other centrifugal membranes did not efficiently recover EVs with 80% reduction in particle concentration and protein concentration measurements below detection threshold due to aspecific adherence of EVs to the centrifugal membranes. Similar findings were observed for plasma and urine, however the differences were less pronounced, probably due to abundant proteins masking centrifugal filter membranes. The Qubit® protein assay kit obtained a respectively 1.5-fold and 2-fold higher protein concentration measurement with the least variance as compared to microBCA and Bradford. The Optiprep™ concentration of EV samples obtained by pelleting density fractions was estimated 1.5–2.5%, whereas no Optiprep™ remnants were detected after EV retrieval from density fractions by size-exclusion chromatography. In addition, removal of Optiprep™ remnants from EV samples improved protein identification by 40-fold as measured by number of unique proteins identified.
Conclusion: The choice of centrifugal filters and protein assay kits as well as residuals of EV isolation media can confound EV analysis and should be carefully considered when performing omics approaches and functional assays.

OT7.03

RNA profiling limits for nanoFACS-sorted extracellular vesicles

Aizea Morales-Kastresana[1], Christopher Grant[2], Peter Choyke[3], Jane Trepel[4], James Gulley[5], Min-Jung Lee[4], Jenn Marte[5], Kevin Camphausen[6], Xiaolin Wu[7], Kenneth Witwer[8], Jay A. Berzofsky[9] and Jennifer C. Jones[9]

[1]National Cancer Institute, National Institutes of Health; [2]National Institutes of Health; [3]Molecular Imaging Branch, NCI; [4]Developmental Therapeutics Branch, NCI; [5]Genitourinary Malignancy Branch, NCI; [6]Radiation Oncology Branch; [7]Leidos Biomedical Research, Inc; [8]The Johns Hopkins University School of Medicine; [9]National Cancer Institute, Vaccine Branch

Introduction: One of the major aims of our research programme is to delineate the molecular profiles of the contents of sorted extracellular vesicle (EV) subsets. The sensitivities of most EV profiling methods (such as RNAseq, NanoString nCounter miRNA Expression Assay, HTG EdgeSeq) are commonly reported as how many microliters of input biofluid are required, rather than how many EVs are required. However, in our studies of sorted EV subsets, it is critical to know how many EVs are required for these profiling methods. In order to systematically delineate the limits of RNA cargo profiling methods for EVs, we performed three different miRNA-omic profiling methods with titrations of EVs, ranging from 10,000 to 1 trillion.
Methods: EVs were isolated by differential ultracentrifugation and size exclusion chromatography. Nanoparticle tracking analysis (NTA) demonstrated that the EVs in this study ranged in size between 70 and 150 nm. NanoFACS was performed with a high-resolution jet-in-air flow cytometer, as previously described, as well as with a next-generation Avalanche Photodiode-equipped instrument. We compared the miRNA signatures of titrated EV populations, using three different RNAseq and high-sensitivity small RNA-omic analysis platforms.

Results: We found a wide range of sensitivity of the RNA-omic profiling methods, in terms of minimum required EV number to observe a robust RNA profile. Not surprisingly, one method that we tested, which does not use any form of amplification, failed to produce a robust profile when fewer than 1–10 billion EVs were used for the assay. On the other hand, next-generation sequencing methods, which used an amplified library, required fewer EVs (as few as 0.5–1 million EVs) to produce a characteristic small RNA profile.
Conclusions: After EV analysis and sorting, the limits of detection of down-stream assays must be well known and wisely selected, in order to optimise the amount of information that can be obtained with the sorted EVs. NanoFACS is a useful method for the identification and isolation of tumour, immune cell and treatment-associated EV subsets, but optimal sensitivity is equally as important for the next-step assays, and amplification may be required for all -omic studies with nanoFACS-sorted EV subsets, or any other -omic studies that seek to profile populations of fewer than 1 billion EVs.

OT7.04

Morphological plasticity of EVs – do some EVs have motility?

Aleksander Cvjetkovic[1], Rossella Crescitelli[1], Cecilia Lässer[1], Davide Zabeo[2], Per Widlund[3], Thomas Nyström[3], Johanna Höög[2]* and Jan Lötvall[1]*

[1]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden; [2]Department of Chemistry and Molecular Biology, University of Gothenburg, Sweden; [3]Department of Microbiology and Immunology, University of Gothenburg, Sweden

*Equally contributing authors.

Introduction: Extracellular vesicles (EVs) are generally thought of as inert information-packages used by cells in intracellular communications. Their shape was previously thought to be round. However, several recent studies have, with the help of cryo-electron microscopy (cryo-EM), shown that there is a great variance in EV morphology. Here we show that some EVs are not necessarily static in their shape, but rather plastic, being able to alter their morphology.
Methods: EVs from yeast, a human mast cell line (HMC-1) and human body fluids were isolated with differential ultracentrifugation. The EVs were then allowed to settle on glass bottom dishes and were subsequently fluorescently dyed with PKH67. They were then visualised with a fluorescence microscope, and time lapse photos were acquired. Moreover, cryo-EM was conducted on EV isolates.
Results: Cryo-EM revealed the presence of elongated EVs in both HMC-1 samples as well as human ejaculate. Some of these EVs contained filamentous structures, reminiscent of actin, in their lumen. Fluorescence microscopy time lapse series showed that a fraction of the vesicles undergo morphological changes within minutes. Most observed events show elongated fluorescent structures round up to spheres. However, EVs also extended protrusions from their main body.
Conclusion: A subset of EVs have the ability to alter their shape. CryoEM suggests that actin dynamics might be a mechanism that allows EVs to shape-change. The ability of EVs to move has a number of implications that could be relevant to both EV biogenesis and uptake. One could also envisage a more directed and active role of EVs in cellular communication than previously assumed.

OT7.05

Microflow cytometry: the Apogee A50 is a sensitive standard tool for extracellular vesicle analyses in liquid biopsies

Desmond Pink, Robert Paproski, Deborah Sosnowski, John Lewis and Catalina Vasquez

University of Alberta, Canada

Detection of biomarkers in liquid biopsy samples is a rapidly expanding field, yet standardised protocols for detection limits have still not been set. Levels of extracellular vesicle (EV) biomarkers in liquid biopsy samples often constitute a very small fraction of the total EVs (<1%). We estimate that in liquid biopsy samples, with most EVs in the 80–200 nm range, there may only be 10's to hundreds of available antibody binding sites for biomarker on each EV. Microflow cytometry analysis of EVs is not trivial, but here we report that quantitative and reproducible detection of these rare biomarker signals on single EVs in complex fluids.
In order to establish parameters for maximal sensitivity and quantitative stability of biomarker signal, we have utilised the optical reporter palmitoylated-EGFP to label membrane EVs in cancer cells as a surrogate biomarker. Conditioned media from healthy LNCaP cells (PALMGFP) was used as a positive signal spike in plasma, serum and urine from healthy volunteers. To mimic the variability in patient EV concentration, PALMGFP was spiked into increasing concentrations of EVs (~10^5–5×10^6 total EVs) from different fluids. To test signal stability and machine reliability, PALMGFP spiked into plasma at high/low levels was aliquoted into 96 samples over 8hrs using an autosampler to test signal stability. Replicate samples were likewise tested for 30 s to 2 min to determine the mean analysis time required to achieve a stable detection rate. All samples were analysed using the Apogee A50, triggering on large angle/small angle scatter.
PALMGFP conditioned cell culture media typically has >10% of the whole sample as GFP positive compared to ~0.1% of standard LNCaP GFP which permits a greater dynamic range for testing. Detection of PALMGFP spike in both serum and plasma was linear from 35–2000 total events when diluted in samples with 10^5–2.5×10^6 background EVs with 100% recovery of the total spike – a 0.001% detection rate. When background EVs reached 5×10^6 events, the analysis was still linear, but recovery was reduced. Single EV analysis was further confirmed by maintenance of light scattering intensity of the positive GFP signal across the dilution. PALMGFP spike into urine was confounded by high levels of fluorescent signal. These are being further optimised. Detection rate of positive PALMGFP signal events in plasma and serum was highly reproducible over 8hrs with 5–5×10^5 EVs). The detection rate of PALMGFP signal was stable after only 30 s of analysis. These tests have been replicated using PSMA, CD9 and CD63 antibodies. To utilise the PALMGFP EV label as a measure of tumour growth, we established PALMGFP tumours in mice and avian embryo models. We have successfully measured PALMGFP EV signal in plasma, and are now validating the EV signature with human leucocyte antigen (HLA-ABC) signal.
We have confirmed that using microflow cytometry, we can detect rare positive signal events that match the expected biomarker levels on EVs in liquid biopsies. Using the Apogee A50 platform EV analysis in complex fluids is fast, yet sensitive, reproducible and can be used to assess disease biomarkers both in the lab and in clinic.

OT7.06

Shotgun proteomic analysis of plasma-derived extracellular vesicles isolated by novel Vn96 peptide, size exclusion chromatography and centrifugation demonstrates the possibility of isolating distinct vesicle subpopulations

Anne Borup[1], Ole Østergaard[2,3], Anders Askeland[1], Niels H.H. Heegaard[2,4], Gunna Christiansen[5], Søren Risom Kristensen[1] and Shona Pedersen[1]

[1]Department of Clinical Biochemistry and Clinical Medicine, Aalborg University Hospital, Aalborg, Denmark; [2]Department of Autoimmunology and Biomarkers, Statens Serum Institute; [3]The Novo Nordisk Foundation Centre for Protein Research, University of Copenhagen, Copenhage, Denmark; [4]Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Odense, Denmark; [5]Department of Medical Microbiology and Immunology, University of Aarhus, Aarhus, Denmark

Introduction: The extracellular vesicle (EV) proteome is of particular interest, as it contains information of diagnostic value and biological function. Nevertheless, EV proteome analysis is challenging due to difficulties in isolating pure EV populations, making the establishment of an effective workflow for EV proteome analysis a top priority. The purpose of this study was thus to compare three different plasma EV isolation methods and their usability for downstream discovery based EV proteome analysis when using tandem mass spectrometry.
Methods: The EV isolation methods included: (1) Centrifugation (18,890*g*), (2) size exclusion chromatography (SEC), and (3) EV precipitation using a peptide (Vn96) that specifically bind to EVs. For EV proteome characterisation, trypsinised EV-isolates were analysed using a Q-Exactive HF. EVs were

characterised using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and western blotting (WB).
Results: EVs were recovered in all isolation methods, confirmed by NTA, TEM and WB. The largest particles were found in centrifugation (~170 nm) followed by subsequently smaller particles in Vn96 (~123 nm) and SEC (~107 nm). Proteomic characterisation identified 1500, 959, and 372 proteins in centrifugation, SEC, and Vn96, respectively. Of these proteins ~96% (centrifugation), ~95% (SEC), and ~91% (Vn96) were EV associated, determined by vesiclepedia and gene ontology (GO) analysis. When compared to specified EV subtype markers proposed by Kowal and colleagues (1).smaller EVs were enriched in SEC while larger EVs were enriched in centrifugation. Vn96 displayed similar enrichment of both small and large EV markers. Additionally, the GO analysis revealed some isolate contamination, where SEC was highly abundant in lipid components while centrifugation was abundant in protein complexes. Vn96 contained minimal contamination. Finally, a strong correlation was seen between APO-B-100 intensity and particle concentration, showing that co-isolation of lipid contaminants affect NTA results.
Conclusion: We have shown that the isolation methods used are capable of isolating different EV proteome fractions, thereby demonstrating that EV isolation method can be chosen based on which EV proteome fraction one wants to study and/or the EV purity needed.

Reference

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Room: Metropolitan Ballroom East
Symposium Session 8 – EV Interactions with Cellular Targets
Chairs: Dolores Di Vizio and Janusz Rak 3:30–5:15 p.m.

LBO.06

Human adipose stem cells originated exosomes improving survival rate of rats with acute liver failure probably by releasing lncRNA H19

Yinpeng Jin and Qingchun Fu

Shanghai Liver Disease Research Center, The 85th Hospital of PLA

Introduction: It has been confirmed that the stem cells promote the regeneration of damaged tissues mainly through the "paracrine effect". As the major carrier responsible for exocytosis of the stem cells, exosome is highly likely to play an important role in stem cell therapy.
Methods: 1. Human adipose-derived stem cells (hASCs) were separated from human adipose tissues and used to prepare hASCs exosomes with modified multi-ultrafiltration concentration method of our research group; scanning electron microscope, Nanosight granulometer and antibody microarrays were employed to identify the morphology, particle size and phenotypes of the hASCs exosomes, and the protein mass spectrometry as well as the second generation sequencing technology used to determine the protein and RNA components in the hASCs. 2. 78 rats with acute liver failure were randomly assigned to 5 groups to receive treatment with the same volume of low concentration (20 μg/rat) and high concentration (100 μg/rat) of hASCs exosomes, low concentration (20 μg/rat) and high concentration (100 μg/rat) of hASCs lysis buffer as well as hASCs (2 x 106 cells/rat) or phosphate buffer solution (PBS), respectively, through femoral vein infusion. The survival rate of rats was observed, and analyses on gene sequencing and signal pathways conducted to explore the potential mechanism of hASCs exosomes in treating the rat models with acute liver failure. 3. 25 rats with acute liver failure rats were randomly assigned to 3 groups to receive the treatment of the same volume of hASCs exosomes (100 μg/rat), hASCs exosomes with silent H19 gene (100 μg/rat) or phosphate buffer solution (PBS), respectively, by femoral vein infusion. The survival rate of the rats was analyzed.
Results: 1. The high-purity hASCs exosomes were collected from the supernatant of hASCs culture using multi-ultrafiltration concentration method, presenting spherical bodies under scanning electron microscopy and Nanosight granulometer with a uniform size of 30-200 nm in diameter; The antibody microarrays indicated high expression of the characteristic markers, such as CD63, CD81, FLOT1, ALIX and ANXA5, on the surface of hASCs exosomes. 2. With protein mass spectrometry and the second-generation sequencing technology, more than 300 types of proteins 2000 kinds of microRNAs were detected in hASCs exosomes. 3. The rat survival curves showed that rat survival rate was 100% and 62.5% respectively in the high concentration and low concentration hASCs exosome group, significantly higher than in the PBS control group (27.3%) ($P < 0.05$); According to the results of gene sequencing for rat liver tissues, hASCs exosome transplant significantly upregulated the genes associated with blood coagulation function and drug metabolism pathways, and dramatically downregulated the genes related to inflammatory responses and chemokine signaling pathways. 6. Signaling pathway analysis revealed an evident upregulation in long chain non-coding RNA (lncRNA) H19 in the liver tissues of rats in hASCs exosome groups, which is likely associated with the upregulation of pathways related to blood coagulation function as well as drug metabolism. A decrease in the rat survival rate to 40% was observed in the rats with acute liver failure when treated with H19 gene silencing hASCs, with a statistical significance as compared with the hASCs exosome groups.
Summary/Conclusion: hASCs exosomes can accelerate the regeneration of the damaged liver cells and improve the survival rate of rats with acute liver failure probably by upregulate the pathways associated with blood coagulation function and drug metabolism as a result of lncRNA H19 release.

Funding: We wish to acknowledge support from the following funding sources: financing for key medical innovation projects of the Nanjing Military (project number: 14ZX01); China Hepatitis Prevention and Treatment Foundation - Tian Qing Liver Research Fund Project (project number: TQGB20150104)

OT8.01

Inspired by nature: characterisation of mechanisms of extracellular vesicle uptake

Helena Costa Verdera[1], Jerney Gitz-Francois[1], Raymond M. Schiffelers[2] and Pieter Vader[1]

[1]University Medical Centre Utrecht; [2]Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, Utrecht, The Netherlands

Introduction: RNA-based therapeutics represent one of the most promising new areas of drug development. Unfortunately, despite recent progress in the development of RNA delivery systems, delivery efficiency of RNA molecules remains unsatisfactory. Recent evidence has established that extracellular vesicles (EVs), including exosomes and microvesicles, form an endogenous transport system through which macromolecules, including RNA, are exchanged between cells. Understanding the biology underlying EV-based intercellular transfer of RNA is of great importance for the development of EV-based delivery vehicles. Here, we sought to characterise the cellular mechanisms involved in EV uptake.
Methods: EVs from A431 cells were isolated using a novel size-exclusion chromatography-based method. Vesicles were analysed by nanosight analysis, western blotting and electron microscopy. Internalisation of fluorescently-labelled EVs was evaluated in HeLa cells, in 2D (monolayer) cell culture as well as 3D spheroids. Uptake was assessed using flow cytometry and confocal microscopy, using chemical and siRNA approaches for inhibition of individual endocytic pathways.
Results: Experiments with chemical inhibitors revealed that EV uptake by HeLa cells depends on cholesterol and tyrosine kinase activity, which are implicated in clathrin-independent endocytosis, and on Na^+/H^+ exchange and phosphoinositide 3-kinase activity, which are important for macropinocytosis. Furthermore, EV internalisation was inhibited by siRNA-mediated knockdown of caveolin-1, flotillin-1, Rac1, RhoA and Pak1, but not clathrin heavy chain and CDC42.
Conclusion: Together, these results suggest that A431 EVs enter HeLa cells predominantly via clathrin-independent endocytosis and macropinocytosis. Identification of EV components that promote their uptake via pathways that lead to functional RNA transfer might allow development of more efficient delivery systems through EV-inspired engineering.

Acknowledgements: PV is supported by a VENI Fellowship (# 13667) from NWO-STW.

OT8.02

Live imaging and biodistribution of 89Zr-labelled extracellular vesicles in rodents following intravenous, intraperitoneal, intrathecal, and intra-cisterna magna administration

Nikki Ross[1], Kevin Dooley[1], Ohad Ilovich[2], Vijay Gottumukkala[2], Damian Houde[1], Emily Chan[1], Jan Lotvall[1] and John Kulman[1]

[1]Codiak BioSciences, MA, USA; [2]InviCRO

Introduction: ^{89}Zr is widely used as a tracer for imaging the biodistribution of monoclonal antibodies, owing to its commercial availability, well-developed radiochemistry and suitability for positron emission tomography (PET). Here we describe a method for ^{89}Zr labelling of

extracellular vesicles (EVs) and demonstrate its application for PET combined with anatomical imaging by X-ray computed tomography (PET/CT).
Methods: EVs were generated from human amniocyte-derived (CAP) cells and human embryonal kidney-derived (HEK) cells, and purified by differential centrifugation and sucrose density gradient ultracentrifugation. Prior to ^{89}Zr labelling, EVs were analysed by SEC-HPLC, western blotting, and electron microscopy. EVs were sequentially treated with p-SCN-Bn-Deferoxamine and $^{89}Zr^{4+}$ to achieve stable ^{89}Zr labelling, and administered to mice by intravenous (IV) and intraperitoneal (IP) routes and to rats by intrathecal (IT) and intra-cisterna magna (ICM) routes. Animals were imaged by PET/CT at several time points up to at least 24 h, and co-registered 3D image reconstruction was performed. Organs were harvested to assess levels of ^{89}Zr-labelled EV accumulation. Selected organs were sectioned and subjected to autoradioluminography.
Results: Biodistribution patterns following IV and IP administration did not significantly differ for EVs of disparate cellular origin (CAP and HEK), but varied greatly as a function of route of administration. The liver and the spleen were the primary sites of uptake following IV administration. Following IP administration, a pattern of punctate thoracic and abdominal distribution was observed, with predominant uptake in the pancreas and spleen. Autoradioluminography revealed EV accumulation within the parenchyma of the pancreas. Following IT administration, broad distribution was observed in the spinal cord and ventricular network of the brain, and more restricted ventricular distribution was observed following ICM administration.
Conclusions: Labelling of EVs with ^{89}Zr is highly suitable for live PET/CT imaging with 3-D image reconstruction, the assessment of biodistribution after organ harvest, and histological evaluation by autoradioluminography. Biodistribution patterns differed greatly as a function of administration route, but not of EV cellular origin.

OT8.03

Determining the fate of extracellular vesicles in C. elegans: trafficking of the released organelle, the post-mitotic midbody

Gholamreza Fazeli, Michaela Geisenhof, Linda Irmisch and Ann Wehman

Rudolf Virchow Centre for Experimental Biomedicine, University of Wuerzburg, Germany

Introduction: Cells release organelles in addition to extracellular vesicles. For example, in animals, the midbody coordinates the end of cytokinesis when two daughter cells separate through an intercellular bridge and the midbody remnant is released extracellularly by abscission on both sides of the bridge. Released midbody vesicles can be found in body fluids, but are often phagocytosed *in vivo*. The fate of phagocytosed midbodies was unclear.
Methods: To determine the fate of midbodies, we used genetics and live imaging to examine the role of phagocytic, endosomal, and autophagic proteins on individual midbodies in developing *C. elegans* embryos.
Results: We first confirmed that midbodies are released in vesicles using the ZF1 degradation tag to label extracellular vesicle membranes. Released midbodies are internalised via actin-driven phagocytosis, which we show requires the RAB-5 GTPase to localise the Class III phosphoinositide 3-kinase (PI3K) complex at the cortex, leading to recycling of the phagocytic receptor CED-1 to the plasma membrane. Further, we found that RAB-5 and RAB-7 appear on midbody phagosomes, directing their maturation similar to endosomes. Proteins normally associated with macroautophagy, such as the Atg8/LC3 homologues LGG-1 and LGG-2, localise around the midbody phagosome and are required for midbody degradation. Additionally, we observed that the Rab2 homologue UNC-108 is required for acidification of the midbody phagosome, demonstrating that LC3-associated phagosomes (LAPosomes) acidify via a common pathway with classical phagosomes.
Conclusion: These studies reveal that internalised midbody remnants are degraded via LC3-associated phagocytosis (LAP). Phagocytosis of midbodies is likely to terminate midbody signalling after their release. The recruitment of LC3 to the phagosome is likely necessary for the degradation of a double membrane-wrapped phagosome. The fate of the midbody reveals how cells cope with released extracellular organelles.

OT8.04

Arginine-rich cell-penetrating peptide-modified extracellular vesicles for improved intracellular drug delivery

Ikuhiko Nakase[1], Kosuke Noguchi[1], Ayako Aoki[1], Tomoka Takatani-Nakase[2], Ikuo Fujii[1] and Shiroh Futaki[3]

[1]Osaka Prefecture University, Osaka, Japan; [2]Mukogawa Women's University, Hyogo, Japan; [3]Kyoto University, Hyoto, Japan

Introduction: Extracellular vesicles (EVs, 30–200 nm), secreted by various cell types, contain bioactive molecules (e.g. microRNAs and enzymes). EVs are anticipated to be the next generation of drug delivery tools owing to their pharmaceutical advantages, including controlled immunogenicity, utilisation of cell-to-cell communication pathways, and modification and encapsulation of functional molecules. For effective intracellular drug delivery by EVs, the efficacy of EV uptake by cells must be improved. In this study, we developed EVs modified with arginine-rich cell-penetrating peptides (CPPs) that actively induce macropinocytosis in cells for enhanced cellular EV uptake and cytosolic release of EV contents.
Methods: All peptides were prepared via Fmoc solid-phase synthesis. Secreted EVs were isolated via ultracentrifugation of HeLa cells stably expressing GFP-fused CD63 (an EV (exosome) membrane marker protein).
Results and Conclusion: We recently found that macropinocytosis (accompanied by actin reorganisation, ruffling of the plasma membrane, and engulfment of large volumes of extracellular fluid) is an important cellular uptake pathway for EVs. We modified EVs using oligoarginine peptides that are arginine-rich CPPs and induce macropinocytosis via the proteoglycan on plasma membranes. Oligoarginine peptide (R*n*, n = 4–16)-modified exosomes were prepared by cross-linking the peptides and EVs via *N*-ε-maleimidocaproyl-oxysulfosuccinimide ester. When we examined the effects of the Rn modification on cellular exosome uptake, the number of arginine residues on the peptide-modified EVs significantly affected both macropinocytosis induction and cellular uptake. To confirm our findings, we artificially encapsulated the ribosome-inactivating protein, saporin, in oligoarginine peptide-modified EVs (saporin-EVs). We found that saporin-EVs modified with hexadeca-arginine (R16) peptides showed effective anticancer activity. Our findings may contribute to the development of EV-based intracellular drug delivery systems.

OT8.05

Surface glycosylation of extracellular vesicles and implications on their interaction with target cells

Joana Gomes[1], Sofia Carvalho[2], Cristina Peixoto[2], Paula Alves[2], Markus Glatzel[3], Manfred Nimtz[4] and Julia Costa[1]

[1]ITQBNOVA; [2]ITQBNOVA, IBET; [3]University Medical Centre Hamburg-Eppendorf, Germany; [4]Helmholtz-Zentrum für Infektionsforschung

Introduction: Glycosylation is a common post-translational modification of proteins from eukaryotes. Cell surface contains glycans that mediate interactions with other cells. Extracellular vesicles (EVs) are rich in glycoconjugates. Here, we aimed at characterising glycosignatures from EVs having in view the investigation of their functional role in uptake.
Methods: EVs were purified from cell supernatants (HEK-293, glioma Tu-2449 cells) by ultracentrifugation, and were analysed by immunoblotting, nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). Membranes (MBs) were obtained by cell sonication and ultracentrifugation. Glycomics included lectin blotting (1), HPAEC-PAD, NP-HPLC and mass spectrometry (2).
Results: EVs were detected with markers, including CD63, CD81, flotillin-2 and LGALS3BP. EVs had heterogeneous size, presented average diameters around 100 nm by NTA, and cup-shape by TEM. Lectin blotting showed that EVs were rich in glycoproteins with α2,3/6-linked sialic acid and fucose among other sugars. Specific N-glycan profiles were found by HPAEC-PAD and NP-HPLC combined with exoglycosidase digestions and mass spectrometry. EVs had predominantly complex glycans with sialic acid and some high mannose glycans, whereas MBs were rich in high mannose glycans. We found that HEK-293 EVs interacted with Tu-2449 cells and the impact of some glycans on the

interaction, based on glycosidase digestion and effect of competitive sugars, will be discussed.
Conclusion: EVs displayed specific glycosignatures, which opened novel perspectives to explore their role in the interaction with other cells.

Acknowledgements: We thank: Linda Streich, GlycoThera, Germany for help with glycan mapping, Dr. Erin Tranfield, Ana Sousa, IGC EM Facility, Portugal for TEM analysis, project ENMed/0001/2013, FCT, Portugal.

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OT8.06

The Amnis imaging stream flow cytometer platform allows discrimination of different vesicles types in mesenchymal stem cell-derived supernatants

Rita Ferrer-Tur[1], André Görgens[1,2], Michel Bremer[1], Kyra de Miroschedji[1,2], Verena Boerger[1], Peter Horn[1] and Bernd Giebel[1,2]

[1]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; [2]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

Human mesenchymal stem/stromal cells (MSCs) represent a promising tool in regenerative medicine and in immune therapy. Up to now, more than 700 NIH-registered clinical trials investigated their therapeutic potential in various diseases. Maybe due to the existence of therapeutically more and less active MSC-subtypes, controversial outcomes have been described for the MSC treatment of several different diseases. As MSCs seem to exert their beneficial therapeutic via extracellular vesicles, we hypothesised that extracellular vesicle (EV) fractions being released by independent MSCs differ in their functional properties. Indeed, by comparing immunomodulatory features of independent MSC-EV fractions, we observe huge differences, e.g. in the capability to supress T cell proliferation of PHA stimulated T cells.
Since we raise MSCs in human platelet lysat (hPL) supplemented media which contain a high proportion of nano-sized vesicles, obtained MSC-EV fractions contain a mixture of non-processed hPL vesicles and EVs secreted by the MSCs. To this end obtained EV fractions should be considered to contain heterogeneous vesicle types. For now, standard analysation platforms such as nanoparticle tracking analyses (NTA), dynamic light scattering (DLS), tunable resistive pulse sensing (TRPS) and western blotting do not allow discrimination of different vesicle types.
In our ongoing experiments we have optimised the analyses of nano-sized single vesicles on the Amnis imaging flow cytometer platform and are now able to characterise vesicles and EVs including exosomes, microvesicles and apoptotioc bodies at the single vesicle level. Upon staining with different antibodies, we indeed can discriminate hPL derived vesicles from MSC released EVs, now. While most hPL vesicles express CD9, MSC-EVs are CD9 negative. However, in contrast to hPL vesicles most MSC-EVs express CD81. By implementing additional antibodies, we can further discriminate different hPL vesicle and MSC-EV subtypes. Amongst others, the optimised analysis platform enables us now to determine the content of hPL vesicles being consumed by independent MSC preparations and the content of the EVs being released by these cells.

Room: Harbour Ballroom
Symposium Session 9 – EV Mediated Communication in Cancer I
Chairs: Peter Kurre and Olga Volpert 3:30–5:15 p.m.

LBO.07

HER2-targeted drug-resistance is associated with immune evasion in cancer cells and their derived extracellular vesicles

Vanesa Martinez[1], Sadhbh O'Neill[1], Josephine Salimub[2], Susan Breslin[1], Aled Clayton[3], John Crown[4] and Lorraine O'Driscoll[5]

[1]Trinity College Dublin, Ireland; [2]Cardiff University, Cardiff, United Kingdom; [3]Division of Cancer and Genetics, School of Medicine, Cardiff University and Velindre Cancer Centre, Cardiff, United Kingdom; [4]St. Vincent's University Hospital, Dublin, Ireland; [5]Trinity College Dublin, Ireland

Introduction: Recent we established that Neuromedin U (NmU) plays a substantial role in HER2-overexpressing breast cancer, correlating with increased aggressiveness, resistance to HER2-targeted therapies and poor survival outcome for patients. Here we aimed to elucidate NmU's mechanism-of-action.
Methods: Drug-sensitive HER2-positive breast cancer cells were engineered to stably over-expressing NmU. In parallel, drug-sensitive cells were exposed to HER2-drugs for ~6 months, to acquire drug-resistance. Approved by SVUH Ethics Committee, serum specimens were procured from consenting patients with HER2-tumours before they received neo-adjuvant treatment with HER2-targeted drugs. Extracellular vesicles (EVs) were isolated from the cultured cells' media and patients' sera using filtration and ultracentrifugation. EVs were characterised by immunoblotting, nanoparticle tracking analysis and electron microscopy. ELISA measured TGF-β_1, PD-L1, IL-2 and IFN-gamma. Flow cytometry analysed PD-L1; CD24/CD44 as markers of stem cells; and apoptosis. Trastuzumab-mediated antibody-dependent cell cytotoxicity (ADCC) was assessed using T cells from PBMC, measuring cell lysis by LDH release.
Results: NmU-overexpressing cells and their EVs have increased immunosuppressive cytokine TFGM-β_1 and lymphocyte activation inhibitor PD-L1. These cells also showed resistance to ADCC, implicating NmU in enhancing immune evasion. All these features were also found in acquired drug-resistant cells that express higher levels of NmU than their drug-sensitive counterparts. Interestingly, EVs from drug-resistant cells transmitted increase levels of TGF-β_1 to drug-sensitive cells. Furthermore, TGF-β_1 levels were significantly higher on EVs from patients who subsequently did not respond to treatment, compared to those who gained some benefit.
Summary/Conclusion: We report a new mechanism-of-action for NmU that enhances resistance to the anti-tumour immune response. Furthermore, EV levels of TGF-β_1 correlating with patients' response versus resistance to HER2-targeted drugs suggests a potential use of EV-TGF-β_1 as a minimally-invasive companion diagnostic for such anti-cancer treatment.
Funding: HRB [HRA-POR-2014-658]; Breast Cancer Now [2015NovSP686]; Irish Cancer Society [CCRC13GAL]; H2020 ME-HaD [BM1202].

OT9.01

Intercellular communication mediated by exosomes as a new therapeutic target for pancreatic cancer

Nuno Bastos[1], Carolina de Freitas. Ruivo[2], Carlos Melo[3], José Machado[1] and Sónia Melo[1]

[1]i3S – Ipatimup; [2]i3S – Instituto de Investigação e Inovação em Saúde; [3]The Gurdon Institute, University of Cambridge, United Kingdom

Cancer treatment experienced significant advance over the last years, mainly due to the development of targeted therapies against key biological pathways. Despite this promising scenario, targeted therapy in pancreatic cancer (PDAC) is still not a reality and the available therapies are limited, with dismal contribution for patient survival. Cell communication, in spite of playing a fundamental role in all steps of tumour progression, is still off the cancer therapy landscape. Exosomes, a subtype of extracellular vesicles, are an important cell-to-cell communication system with neighbour and/or distant cells. Exosomes are derived from the endocytic pathway and formed within multivesicular bodies (MVBs). Our main aim is to study the role of exosomes biogenesis during pancreatic cancer progression and understand if targeting cancer exosomes biogenesis could be a new therapy avenue in pancreatic cancer. Rab GTPases are crucial proteins in exosomes biogenesis and are involved in all stages of the endocytic pathway. We show that during pancreatic cancer progression Rab-5, -7, -27a and -27b are differently expressed. Increased expression of Rab-27a and -27b correlates with an increase in exosomes number, and these features are associated with a more aggressive phenotype. In contrast, Rab-5 and -7 do not show correlation between their protein levels and the number of exosomes released. Additionally, when treated with gemcitabine, the standard care chemotherapeutic for pancreatic cancer, cancer cells change their exosomes biogenesis pattern, increasing exosomes release. Finally, we are using an inducible and conditional genetically engineered Rab-27a knockout mouse model, crossed with a mouse model that spontaneously develops PDAC, to study the function of exosomes and its biogenesis in disease progression and therapy response, and to evaluate exosomes-mediated communication as a new therapeutic option in pancreatic cancer.

OT9.02

Exosomes from bovine milk reduce the tumour burden and attenuates cancer cachexia

Monisha Samuel[1], Markandeya Jois[1] and Suresh Mathivanan[2]

[1]Department of Physiology, Anatomy and Microbiology, La Trobe University, Victoria, Australia; [2]La Trobe Institute for Molecular Science, Victoria, Australia

Introduction: Milk has long been associated with good health and is one of the most consumed beverages throughout the world. Exosomes are 30–150 nm membranous vesicles of endocytic origin that are released by all cell types and are also detected in bodily fluids including milk. Whether these milk-derived exosomes can serve as cross-species messengers and have a biological effect on host organism has been poorly understood. Here, we examined the stability of bovine milk exosomes in degrading conditions and studied their biodistribution using mouse models and IVIS imaging after oral administration. We also unravel the role of bovine milk derived exosomes in colon cancer progression.
Methods: Milk exosomes were isolated using differential centrifugation and OptiPrep™ density gradient centrifugation. They were further characterised and examined for stability under harsh conditions using western blotting and nanoparticle tracking analysis. IVIS imaging system was used to study the biodistribution of these exosomes on oral gavaging. Mice models were used to understand the role of milk exosomes in cancer progression.
Result: On examining the stability of bovine milk exosomes in harsh conditions, it was concluded that these exosomes are remarkably stable in both acidic and high temperature conditions while colorectal cancer cell-derived exosomes were not. Next, we studied the biodistribution of bovine milk exosomes which suggested that orally administered milk exosomes can survive the harsh intestinal environment and can be trafficked to various organs. Interestingly, after 24 h, the milk-derived exosomes reached multiple organs including liver and spleen in the mice. To understand the role of milk-exosomes in cancer progression, *in vivo* mouse models implanted with colorectal cancer were orally administered with milk-derived exosomes. Remarkably, exosomes isolated from both raw and commercial (grocery store) milk significantly reduced the tumour burden. Furthermore, orally administered milk

exosomes prolonged the survival of the mice by inhibition of tumour-induced weight loss in cancer cachexia mice models.
Summary: Thus this study provides new insights on the significance of milk exosomes in context of mammalian physiology as well as prompt their use as drug delivery vehicles in therapeutic interventions.

OT9.03

Oligodendroglioma cells communicate with neighbouring tumour and normal neural cells via extracellular vesicles

Lata H. Adnani[1], Christian Perotti[2], Jennifer Chan[2] and Carol Schuurmans[1]

[1]Sunnybrook Research Institute, University of Toronto and Biochemistry and Molecular Biology Department, University of Calgary, Canada; [2]Hotchkiss Brain Institute, University of Calgary, Canada

Gliomas are malignant brain tumours comprised of abnormal glial-like cells with stem cell-like properties. *In vitro* screens have identified numerous drugs with activity against glioma cells, yet their *in vivo* effects are frequently disappointing. The discrepancy between *in vitro* and *in vivo* data may be due in part to the inability of *in vitro* screens to recapitulate disease complexity, including interactions between cancer cells and other cells in the microenvironment. Consistent with the importance of homotypic and heterotypic cell interactions in the tumour micro-environment, we found that oncogenes such as *RasV12* and *bRafV600E* act both cell autonomously and non-cell autonomously to confer an abnormal glial cell phenotype in mouse glioma models. Here we assessed the role of extracellular vesicles (EVs) in intercellular communication in a patient-derived oligodendroglioma cell line (BT88). To first assess whether BT88 cells secrete factors that influence tumour-tumour cell interactions, BT88 cells were plated at clonal density in fresh and BT88-conditioned media (CM). Strikingly, BT88-CM inhibited the formation of BT88 tumorspheres. In contrast, BT88-CM promoted the proliferation and sphere-forming capacity of embryonic day (E) 13.5 mouse neural stem cells (mNSCs). To determine whether these effects were EV-mediated, we first used a Cre-based reporter line to demonstrate that BT88 cells secrete EVs that transfer bioactive molecules to neighbouring cells, in this case, the cre recombinase. Next, we targeted the neutral sphingomyelinase 2 (nSMase2) pathway of EV synthesis using a shRNA to *Smpd3*, which encodes for this enzyme. Knockdown of *Smpd3* in BT88 cells reduced tumorsphere number, indicating that EVs are responsible for the inhibitory effects that BT88 cells have on one another. Currently, we are studying whether BT88 cell interactions with mNSCs is also EV-mediated. Taken together, our data supports the idea that EVs mediate at least some aspects of cell-to-cell communication in oligodendroglioma.

OT9.04

HOTAIR affects bladder cancer epithelial-to-mesenchyme transition through both the Canonical WNT-pathway and extracellular vesicles

Claudia Berrondo[1], Thomas Osinski[1], Jonathan Flax[2], Samuel Richheimer[2] and Carla J. Beckham[2]

[1]URMC; [2]University of Rochester, NY, USA

Introduction: Previously we showed the long non-coding RNA Hox antisense intergenic transcript (HOTAIR) is enriched in urothelial bladder cancer (UBC) cell lines, extracellular vesicles (EVs), patient tumours and urinary EVs. Importantly, HOTAIR affects genes involved in epithelial-to-mesenchyme transition (EMT). Loss of HOTAIR correlates with reduced *in vitro* migration and invasion. Several genes affected by HOTAIR are in the Wnt-pathway. HOTAIR facilitates EMT via the Wnt-pathway in several tumours. We show that HOTAIR is necessary for Wnt-responsiveness and its expression increases with Wnt activation. EMT is also regulated through intercellular communication by EVs. HOTAIR regulates thousands of genes. We discovered that HOTAIR knockdown cells produce fewer EVs with altered protein cargo and do not facilitate migration or invasion. Targeting HOTAIR therapeutically may affect EMT through the Wnt-pathway and EVs function.
Methods: LiCl or rWNT treated UBCs gene expression was analysed by qRTPCR, western blot and immunohistochemistry. Scratch and 3D spheroid invasion assays measured *in vitro* EMT. shRNA or siRNA against HOTAIR were used. WNT target and antagonist gene expression was measured by qRT-PCR. Sequencing revealed TCF7L2 binding sites in the promoter region of HOTAIR. siRNA against TCF7L2 or beta-catenin reduced HOTAIR expression. EVs isolatedd by ultracentrifugation and sucrose gradient were analysed using Nanosight. LC MS/MS mass spectrometry and western blot were used to analyse EVs protein.
Results: TCGA data reveals WNT-pathway genes are affected in UBC. LiCl or rWNT treated UBCs have increased EMT related gene expression. rWnt facilitates *in vitro* migration and invasion dependent on HOTAIR. Reduced HOTAIR correlates with decreased WNT-target and increased antagonist gene expression. Importantly, HOTAIR is a target of canonical WNT signalling. Reduced HOTAIR expression affects UBC EV number, content and *in vitro* migration and invasion.
Conclusions: The canonical WNT-pathway is critical in UBC and is functionally dependent on HOTAIR. Therapeutic targeting of the WNT-pathway may affect UBC tumour progression through loss of HOTAIR as loss of HOTAIR affects hundreds of genes that results in reduced EVs number, content and *in vitro* migration and invasion.

OT9.05

Oncolytic adenoviruses encapsulated into the extracellular vesicles as carriers for targeted drug delivery

Mariangela Garofalo[1], Heikki Saari[1], Elisa Lazaro-Ibanes[2], Petter Somersalo[1], Laura Aksela[3], Cristian Capasso[4], Matti Jalasvuori[5], Vincenzo Cerullo[4], Paolo Ciana[6], Lukasz Kuryk[4] and Marjo Yliperttula[1]

[1]Division of Pharmaceutical Biosciences and Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland; [2]Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Finland; [3]Orion Corporation; [4]Laboratory of Immunovirotherapy, Division of Pharmaceutical Biosciences and Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland; [5]Biological and Enviromental Science, University of Jyväskylä, Finland; [6]Division of Oncology and Onco-Haematology, University of Milan, Italy

Introduction: Lung cancer is a highly invasive and rapidly metastasising cancer type. Although many kinds of treatment have been developed during the past decades there is still a lack of effective therapy, since it is still diagnosed at the end-stage of the disease and associated with poor prognosis. Therefore new treatment strategies are in high demand. Efficient anticancer agent and its targeted delivery into the tumour mass is a key prerequisite for the successful cancer therapy. Oncolytic virotherapy is emerging as a potential approach to treat cancer, using viruses, which are specifically engineered to selectively infect, replicate in and kill cancer cells without causing damage to normal cells. However this approach has also disadvantages like low efficacy and production of neutralisation antibodies against virus. Additionally oncolytic viruses are administered intratumorally, thus many solid tumours cannot be treated using this approach. Extracellular vesicles (EVs), which are naturally occurring cargo delivery agents, have a potential to be used as vehicles for drug delivery. Therefore EVs can be used for targeted delivery of the therapeutic agents into the tumour cells and to finally decrease drug toxicity. For these reasons we hypothesised that oncolytic adenoviruses encapsulated into EVs loaded with chemotherapeutic drugs should enhance specific drug delivery for tumour targeting, and thus improve efficacy of cancer treatment.
Methods: Electron and confocal microscope were used to check the encapsulation of adenovirus into EVs, while fluorescent microscope was used to test the EV–virus complex for the functional cell viability assay. The *in vivo* efficacy of EV–virus–drug complex was tested in Balb/c nude mice after intravenous injection.
Results and Conclusions: We found by electron and confocal microscope that oncolytic adenoviruses are encapsulated into EVs. EV–virus and EV–virus–paclitaxel complexes were able to enhance cell death and transduction efficacy in lung cancer (A549) cell line, while *in vivo* efficacy studies showed that tested platform was able to control tumour growth after intravenous injection. Our findings support the idea that an oncolytic adenovirus encapsulated into EVs loaded with therapeutic agents could be used as anticancer drug treatment.

OT9.06

TGFBR2-dependent alterations of exosomal cargo and functions in DNA mismatch repair-deficient colorectal cancers

Fabia Fricke, Jürgen Kopitz and Johannes Gebert

Department Applied Tumour Biology, Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany; Department Cancer Early Detection, German Cancer Research Centre (DKFZ), Heidelberg, Germany

Introduction: DNA mismatch repair-deficient (dMMR) colorectal cancers (CRCs) exhibiting the microsatellite unstable (MSI) phenotype represent about 15% of all CRCs. These tumours show a high frequency (>90%) of inactivating frameshift mutations in the tumour suppressor transforming growth factor beta receptor type 2 (TGFBR2). How altered TGFBR2 signalling affects communication between tumour cells and their environment has not been resolved. Here, we report on MSI-specific molecular and proteomic alterations of exosomes shed by dMMR cells and resulting effects on potential target cells.
Methods: Exosomes were isolated and characterised by electron microscopy, nanoparticle tracking, and western blot analysis. TGFBR2-dependent effects on exosomes were analysed in a MSI CRC cell model system (HCT116-TGFBR2) enabling inducible TGFBR2 expression/signalling. Microsatellite frameshift mutations of exosomal and cellular DNA were determined by PCR-based fragment analysis and protein profiles examined by mass spectrometry. CFSE-labelled exosomes were used to confirm uptake. Exosomal effects on cytokine profiles of recipients were analysed by Luminex and ELISA.
Results: Coding microsatellite frameshift mutation types and pattern in TGFBR2 and other MSI target genes were found to be shared by MSI tumour cells and derived exosomes. Reconstituted TGFBR2 expression and signalling in HCT116-TGFBR2 cells uncovered two exosomal protein subsets specifically originating from TGFBR2-deficient (n = 14) or TGFBR2-proficient (n = 5) donor cells. Uptake of MSI tumour cell exosomes by HepG2 cells was confirmed by confocal microscopy and caused significant alterations of cytokine secretion levels in a TGFBR2-dependent manner (>1.5-fold) predominantly affecting IL-4 (2-fold), stem cell factor (2.5-fold) and platelet-derived growth factor-B (6-fold).
Conclusion: Our results point to a biological activity of MSI tumour cell derived exosomes on recipient cells. These effects are influenced by TGFBR2 signalling in the donor cell, which was also found to impact the exosomal proteome. Since the molecular MSI phenotype of these cells is mirrored in their exosomal DNA, exosomes might facilitate molecular MSI tumour diagnostics complemented by specific exosomal protein markers linked to the donor cell expression status of TGFBR2.

Poster Session PT01 – From Biogenesis to Targeting
Chairs: Frederik Verweij and Vandhana Muralidharan-Chari 5:15–6:30 p.m.

PT01.01

Role of extracellular vesicles in thyroid folliculogenesis

Jonathan Degosserie and Christophe E. Pierreux

de Duve Institute, Université Catholique de Louvain, Belgium

Introduction: Intercellular communication is essential for biological processes such as cellular differentiation and pathological processes such as cancer. Our lab has recently shown that reciprocal communication between epithelial and endothelial cells is of major importance for pancreatic and thyroid organogenesis during murine development. In the developing thyroid, epithelial cells first secrete huge amount of VEGFa that stimulates recruitment of endothelial cells. In turn, recruited endothelial cells invade the thyroid epithelial bud and induce thyroid progenitors to reorganise and form thyroid follicles.
Methods: Using an original *ex-vivo* thyroid culture system that faithfully reproduces *in vivo* thyroid development and follicle formation, we demonstrated that medium conditioned by endothelial cells stimulate folliculogenesis. In addition, this folliculogenic activity could be further purified by high-speed centrifugation of the conditioned medium in a sedimentable material. Morphological and biochemical characterisation of this material lead us to identify round shape membrane structure with an average size of 100 nm and a density of 1.10 g/mL corresponding to extracellular vesicles (EVs). EVs have been recently identified as sophisticated vehicles, containing soluble proteins and nucleic acids, and involved in short and long distances communication processes.
Results and Conclusion: Mass spectrometry analysis of the EVs uncovered the presence of specific vesicular markers as well as of abundant laminin a1, b1 and g1 peptides. EVs purified from endothelial cells pre-infected with laminin a1 shRNA have no folliculogenic activity, indicating that laminin present in the sedimentable material is required for the folliculogenic activity. Our current working hypothesis is that laminins are important for EVs targeting and incorporation in thyroid progenitor cells.

PT01.02

Shuttle mechanisms of extracellular vesicle-enclosed bioactive molecules in ex-vivo and in-vivo systems

Thamara Dayarathna[1], Andrew Chun-Him Poon[2] and Hon S. Leong[3]

[1]LHRI, St. Joseph's Health Care; [2]University of Western Ontario, Canada; [3]Western University, Ontario, Canada

Introduction: Communication between cells is essential for life and survival in multicellular organisms. Apart from signal transduction via chemical transmitters produced by paracrine, endocrine, neurocrine and lumencrine signalling, extracellular vesicles (EVs) play a crucial role in information exchange, particularly in the form of mRNA, protein, bioactive molecules and carbohydrates. However, the total microparticle fraction of EVs in the extracellular matrix (ECM), their roles and mode of action are poorly understood among molecular biologists. Here we present cell-specific EV fraction identification and cell-to-cell shuttle mechanisms of EV-enclosed biomolecules that contribute to various cellular functions including cell differentiation, organ development and cell death.
Methods: EV fractions of endothelial, platelet, leucocyte and erythrocyte were isolated using their explicit protein markers and analysed by nanoscale flow cytometry. To understand the targeting organelles in receiving cells and cellular uptake mechanisms, benign prostatic hyperplasia (BPH) cells were treated with purified EVs stained with SYTO® RNASelect™ specific for RNA. Micro RNA-enriched EV uptake by receiving cells, EV localisation post-uptake, and their time-dependent release from newly received cells were examined and captured by confocal microscopy. Furthermore, lactate dehydrogenase (LDH) content of BPH cells was measured after incubation with purified EVs released from BPH cells which were treated with the cytotoxic agent dimethyl fumarate.
Conclusion: Light scatter plots of nanoscale flow cytometric analysis revealed tetraspanin-specific exosome markers and enriched EV fractions of cell-specific origins. Endothelial, platelet, leucocyte and erythrocyte enriched EV fractions displayed a clear difference in both size and the amount of EVs within the heterogeneous population of healthy human plasma. Cellular fragments or EVs shed by healthy cells contain visible RNA fragments and target to the cell membrane or in the cytoplasm, to specific organelles like the mitochondria and nucleus. Studying components in organelle-specific EV fractions will be the next target on elucidating their physiological functions.

PT01.03

***In vivo* biodistribution of CTX human neural stem cell derived exosomes delivered by various routes of administration**

Patrick Joseph Walters, Caroline Hicks and Randolph Corteling

ReNeuron, Bridgend, United Kingdom

Introduction: ExoPr0, is an exosome product derived from CTX (CTX0E03), a human neural stem cell line currently under clinical evaluation for stroke and limb ischaemia. Using a defined good manufacturing process scalable production of clinical grade cells and their extracellular vesicle products demonstrate batch to batch consistency. ExoPr0 product isolated and purified by tangential flow filtration from spent conditioned media collected during the CTX manufacturing process show reproducible product specification in validated purity and identity tests. We have identified the potential for ExoPr0 as a drug delivery vehicle and as a novel therapeutic product realised by its potential to modulate fibroblasts, immune cells and glioblastoma cell lines in various assays *in vitro*.
Methods: In this study we evaluated the biodistribution of ExoPr0 *in vivo* using the most common clinical and disease applicable routes of administration identified for this product. ExoPr0 was fluorescently labelled prior to administration and detected by optical imaging and histology methods.
Results: Local administration showed tissue specific cell uptake of ExoPr0. In the brain astrocytes in the corpus collosum demonstrated specific targeting and uptake following migration of ExoPr0 from the site of injection in the striatum. In the skin fibroblasts demonstrated uptake of ExoPr0, distribution was also seen to mononuclear and cells of dendritic morphology of lymph nodes draining the injection site. In contrast systemic delivery by the intravenous route resulted in the highest accumulation of ExoPr0 in the liver and bladder. Imaging and histological evaluation of organs confirmed the presence of ExoPr0 in the brain, spleen, lungs and kidneys.
Conclusion: These studies demonstrate the ability to target ExoPr0 to specific tissues and organs. This together with the tissue specific activity of ExoPr0 suggests there is great potential to develop this product for the treatment of more than one disease.

PT01.04

Amoeboid cancer cells shed extracellular vesicles enriched with nuclear derived material

Mariana Reis Sobreiro[1], Jie-Fu Chen[1], Samantha Morley[1], Sungyong You[1], Kenneth Steadman[1], Navjot Kaur Gill[2], Gina C-Y Chu[1], Leland W.K. Chung[1], Hisashi Tanaka[1], Wei Yang[1], Amy C. Rowat[2], Hsian-Rong Tseng[2], Edwin M. Posadas[1], Dolores Di Vizio[1] and Michael R. Freeman[1]

[1]Cedars Sinai Medical Center, CA, USA; [2]University of California, Los Angeles, CA, USA

Please see OPT01.01

PT01.05

Discrete biogenic vesiculation pathways reside malignant and non-malignant breast cells

Jack Taylor and Mary Bebawy

The Graduate School of Health, The University of Technology Sydney, Sydney, Australia

Introduction: Microparticle (MP) biogenesis occurs following cellular activation and follows loss of membrane phospholipid asymmetry and activation of calcium dependent cytosolic cysteine protease activity. MPs confer the transfer and acquisition of cell phenotypes through the intercellular transfer of bioactive molecules. In the context of cancer, Bebawy and colleagues (1) discovered that MPs provide a "non-genetic" mechanism for the acquisition of multidrug resistance and increased metastatic capacity in cancer cell populations.The aim of this study was to define the biogenic pathways involved in MP vesiculation in malignant and non-malignant cells using high resolution biological atomic force microscopy (AFM) (Nanowizard, JPK Instruments, Germany). Identification and elucidation of cancer specific biogenic pathways would provide novel therapeutic targets and strategies to circumvent deleterious traits acquired through MPs in cancer.
Methods: A comparative analysis was performed using non-malignant human brain endothelial cells (HBEC-D3), human mammary epithelial cells (MBE-F), and drug sensitive and resistant human breast adenocarcinoma cells, MCF-7 and MCF-7/Dx respectively. Vesiculation of resting cells and cells activated with calcium ionophore, A23187, were studied ± calpain inhibitor II (ALLM). Cell surface topography and the extent of vesiculation was determined using contact mode methodology.
Results: At rest, malignant cells exhibit an intrinsically higher degree of vesiculation relative to non-malignant cells. In the presence of ALLM, vesiculation was inhibited in malignant cells whilst non-malignant cells exhibited enhanced vesiculation. The latter supports the presence of a calpain independent pathway for vesiculation of normal cells at rest. Increasing intracellular calcium release with A23187 resulted in an increase in vesiculation across all cell types, however this was especially pronounced in non-malignant cells.
Conclusion: We conclude that vesiculation at rest in malignant breast cells is driven by a calcium-calpain dependent pathway, whereas, an alternative pathway governs MP biogenesis in resting normal cells. These results support therapeutic approaches to selectively target malignant cells.

References

1. Bebawy et al., *Leukemia*. 2009; 23: 1643–1649.

PT01.06

Identifying intrinsic components that regulate the secretion of stroma-activating exosomes in prostate cancer

Vincent Yeung[1], Mark Gurney[1], Zsuzsanna Tabi[1], Rachel Errington[1], Jason P. Webber[1] and Aled Clayton[2]

[1]Cardiff University, Cardiff, United Kingdom; [2]Division of Cancer and Genetics, School of Medicine, Cardiff University and Velindre Cancer Centre, Cardiff, United Kingdom

Introduction: Exosomes originate in multivesicular endosomes, and are expelled into the extracellular space to mediate a host of pro-tumourigenic effects. We have previously demonstrated activation of stromal cells within the tumour microenvironment, to a disease-associated myofibroblast-like phenotype, in response to prostate cancer exosomes. Secreted exosomes are, however, heterogeneous in terms of biophysical and molecular properties. Multiple parallel pathways coexist and give rise to this exosome heterogeneity, and the exact pathway for generating exosomes with disease-promoting function remains unclear. Here, we investigated the roles of six proteins (CD9, Rab5a, Rab11b, Rab35, VAMP7 and VPS25) in the generation of stroma activating exosomes.
Methods: Lentiviral-delivered shRNAs were used to knockdown these targets in prostate cancer cells (DU145). Vesicle concentrates were characterised by NTA, western blot and plate-based assays. Fibroblasts were stimulated with cancer cell conditioned media, or vesicle concentrates and their ability to differentiate into alpha-smooth muscle actin positive myofibroblasts was determined by immuno-fluorescent microscopy. Fibroblast/myofibroblast functionality was determined using *in vitro* vessel formation and 3D invasion assays.
Results: Knockdown of Rab11b or Rab35 resulted in a modest attenuation of exosome secretion (~20% by NTA). The remaining vesicles (~80%) exhibited a distinct protein profile, and were insufficient in number or composition to trigger fibroblast differentiation, angiogenesis or mediate pro-invasive behaviour within tumour:stroma spheroids.
Conclusion: Rab11b or Rab35 regulate distinct exosome secretion pathways, but generate essential sub-populations for triggering a cancer-associated fibroblast phenotype. Targeting these elements may offer novel modalities to limit tumour promoting stromal influence in the cancer microenvironment.

PT01.07

The Pseudomonas quinolone signal drives outer membrane vesicle biogenesis in *Pseudomonas aeruginosa*

Catalina Florez, Julie E. Raab, Adam C. Cooke and Jeffrey W. Schertzer

Binghamton University, NY, USA

Introduction: Quorum sensing, the phenomenon of cell-to-cell communication in bacteria, induces virulence and promotes human disease. An important quorum sensing signal in *P. aeruginosa* is the Pseudomonas quinolone signal (2-heptyl-3-hydroxy-4-quinolone, PQS). In addition to signalling, PQS mediates its own packaging and transport between cells by stimulating outer membrane vesicle (OMV) formation. It has been shown that 85% of PQS produced is found in OMVs, demonstrating that these vesicles are the transport vehicle of PQS. We proposed the "bilayer-couple" model for OMV formation, a biophysical model where PQS intercalates into the outer membrane resulting in the induction of membrane curvature. We hypothesise that in accordance with the bilayer-couple model, PQS must be transported from its place of synthesis, the cytoplasm, to the outer cell surface before it can initiate OMV formation.
Methods: We examined two strains of *P. aeruginosa* under different growth conditions to investigate PQS export and to correlate this with OMV formation. PQS was extracted with ethyl acetate and separated and visualised on a thin-layer chromatography plate. OMVs were isolated by ultracentrifugation and were quantified by lipid and nanoparticle tracking analyses. Cellular membranes were separated using sucrose density gradients.
Results: We identified significant strain- and growth medium-dependent differences in the extent of PQS export. Conditions giving rise to the most PQS export also resulted in the greatest level of OMV production. We found that PQS export phenotypes are independent of growth phase and stable throughout the lifecycle of the bacterial culture. We further discovered that cell associated PQS under poor-OMV- producing conditions was largely localised to the inner membrane.
Conclusion: These results suggest that diminished OMV biogenesis is a consequence of failure to export PQS to the outer membrane. We conclude that OMV formation is correlated to the amount of PQS *exported* from the cell (rather than simply to the amount of PQS produced) and that exporting ability is independent of growth phase. These results are consistent with the bilayer-couple model and underscore the possible presence of dedicated PQS export machinery involved in the mechanism of OMV formation.
Funding: NIH.

PT01.08

BAG6 regulates the release of a subgroup of endosomal-derived extracellular vesicles

Maximiliane Schuldner[1] and Elke Pogge von Strandmann[2]

[1]Department I of Internal Medicine, University Hospital of Cologne, Cologne, Germany; [2]Experimental Tumour Research, Centre for Tumour Biology and Immunology, Clinic for Haematology, Oncology and Immunology, Philipps University, Marburg, Germany

Extracellular vesicles (EVs) are increasingly recognised as intercellular mediators by functionally transferring biomolecules to recipient cells.

Depending on their composition, EVs can have either pro- or anti-cancer activity playing a role in diverse steps during tumourigenesis. Recently, our group has identified the multifunctional chaperone BAG6 as a negative regulator of an ESCRT-mediated release of EVs in HEK293 cells (unpublished data). In this project, the melanoma cell line B-16V is used to investigate whether tumour cell-derived EVs are characterised by the expression of BAG6 and/or BAG6-recruited molecules and whether these EVs are taken up by immune cells modulating the anti-tumour immune response.
First experiments showed that CRISPR-Cas9 generated BAG6KO B-16V cells release an increased amount of EVs compared to wild type cells. This phenomenon is reminiscent to human BAGKO cell line HEK293. Strikingly, mass spectrometry of BAG6KO EVs released from hypoxia-stressed B-16V cells revealed a de-regulated expression of vesicle-associated proteins compared to wild type EVs. This specific protein profile most prominently included the up-regulation of ESCRT components and might correspond to a BAG6-regulated subgroup of endocytically derived EVs. Moreover, differential expression of proteins with tumourigenic and angiogenic activity including several integrin subunits was observed.
In order to investigate the significance of BAG6-regulated EVs, a Cre-loxP system visualising EV uptake by recipient cells will be applied. Preliminary experiments have validated the feasibility of the system using *in vitro* co-cultures of cre-expressing B-16V clones and primary reporter spleen cells. Future experiments will focus on the *in vivo* identification of recipient cell types of BAG6KO and wt EVs and how these recipient cells, in turn, are modulated on the molecular level thereby influencing tumour progression.

PT01.09

Kinetics of extracellular vesicle secretion in relation to hyaluronan synthesis

Kai Härkönen[1] and Kirsi Rilla[2]

[1]Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland; [2]Faculty of Health Sciences, School of Medicine, Institute of Biomedicine, University of Eastern Finland

Extracellular vesicles (EVs) have attracted extremely increased interest as a research topic during the recent years. Because of a huge rush to reveal the most fascinating features of these tiny membrane bubbles, many critical steps at basic research may have been overshadowed. One of those steps that have been neglected is the kinetics of vesicle secretion in cell cultures. Factors like cell seeding density, growth rate and time between seeding and vesicle isolation should be taken into account while optimising vesicle isolation protocols.
We have recently shown that activity of hyaluronan synthesis induces shedding of EVs. hyaluronan (HA) is the most abundant glycosaminoglycan of the extracellular matrix, and one of the key components of the niche that promotes renewal of cells and tissues in health and disease. The observed connection between HA and EVs is revolutionary, but the more detailed mechanisms have not been elucidated so far. Activity of HA synthesis is strictly controlled, and cell density and contact inhibition are among the factors that regulate the HA secretion rate.
The aim of this work was to correlate the kinetics of EV shedding with HA synthesis activity in cell cultures. We utilised breast cancer cell lines with naturally high (MDA-MB-231) and low (MCF-7) HA secretion levels. To manipulate HA synthesis activity of these cells, inducible overexpression of HA synthase 3 and a specific inhibitor for HA synthesis, 4-methylumbelliferone were used.
Extracellular vesicles were isolated from cell culture media with ultracentrifugation at different time points after seeding and their concentrations were analysed with nanoparticle tracking analysis (NTA). Levels of HA in the same samples were measured with specific enzyme-linked sorbent assay (HA-ELSA) and correlated with the EV secretion levels and cell counts in the cultures.
This study provides novel information about the kinetics of vesicle secretion in cell cultures and its relation to the activity of HA synthesis. The results suggest that continuous monitoring of the EV yield is important when isolations are performed. The results also show that cell density and growth phase have a strong impact on EV release, suggesting that the kinetics of EV release is highly dynamic and strictly regulated.

PT01.10

Fractionation of discrete extracellular vesicle sub-populations reveals distinct RNA profiles and distinct mechanisms of sorting

Jeremy Henderson, Matthew Shurtleff and Randy Schekman

UC Berkeley, CA, USA

Cells release an array of extracellular vesicles (EVs) consisting of a lipid bilayer and transmembrane proteins enclosing soluble cellular content, including RNA and proteins, and encompassing a broad size range (~30 nm to 1000 nm). By using differential velocity centrifugation coupled with buoyant density flotation we were able to separate two distinct EV sub-populations in MDA-MB-231 cells. The densities of the two sub-populations were 1.07–1.10 g/ml and 1.13–1.15 g/ml for the low-density (LD) and high-density (HD) sub-populations respectively. Immunoblots for soluble and membrane EV markers in the linear gradient, showed a differential distribution, having classical EV markers such as CD63, Alix, Tsg101 present only in HD.
We next probed the RNA content of the distinct vesicle populations. The amount of RNA was similar for LD and HD sub-populations, however the RNA species varied among the sub-populations. Bioanalyzer traces, later confirmed by sequencing experiments, showed that the LD RNA is predominantly tRNA, whereas the HD is also enriched in small RNAs such as miRNAs. At analysing selected miRNAs in deeper detail, we showed that whereas HD miRNAs can display a great enrichment compared to cell lysates (in several cases over 100 fold enrichment), the LD miRNAs don't show enrichment compared to cell lysates, and most of them show the opposite pattern (depletion compare to cells). These observations lead us to think that there is a selective sorting mechanism responsible for packaging miRNAs in HD, but such mechanism is absent in LD, being the LD miRNAs the result of random sampling of cellular RNAs. *In vitro* packaging of miRNAs into exosomes, developed in our laboratory previously, showed that the MDA-MB-231 specific enriched miRNAs are efficiently packaged in this reaction and that their packaging is independent of YBX1, an RNA binding protein found to be essential for packaging miRNAs in HEK 293T derived EVs. This suggests that other mechanisms of sorting miRNAs into EVs play a role in MDA-MB-231 cells, and ongoing experiments are trying to depict them.

Poster Session PT02 – EV Isolation
Chairs: Cecilia Lasser and Jan van Deun 5:15–6:30 p.m.

PT02.01

A rigorous method for exosome isolation from tissue

Laura J. Vella[1], Benjamin J. Scicluna[2], Lesley Cheng[2], Kevin J. Barnham[1] and Andrew F. Hill[2]

[1]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Victoria, Australia; [2]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Victoria, 3084, Australia

Introduction: Understanding the role of exosomes in the brain is a fundamental scientific objective with clinical relevance. Realisation of this goal, however, has been hampered by an inability to isolate genuine exosomes from the brain. Relative to the routine isolation from extracellular fluids, many technical issues must be overcome to successfully isolate exosomes from solid tissue. Exosomes share many physical and molecular properties with other vesicles imposing important limitations. Cell integrity needs to be maintained to minimise co-isolation of particles masking as exosomes and rigorous characterisation needs to be undertaken to confirm enrichment of exosomes relative to exosome mimetics. Here we have taken a critical approach to the enrichment and characterisation of exosomes from human frontal cortex and mouse tissue.
Methods: Vesicles were isolated from human (frontal cortex, Alzheimer's disease or neurological control) or mouse (whole) brain tissues (n = 50 human/n = 30 mouse) and systematically assessed for morphology, density, size distribution and proteomic and genomic content to validate the approach and fulfil the experimental requirements as to be defined as exosomes.
Results: Immunoblot, electron microscopy, proteomics, size distribution, RNA and density gradient analysis confirmed successful isolation of endosome derived exosomes (enriched for syntenin, tsg101 and CD81) from brain tissue. Upon comparing exosomes from Alzheimer's disease (AD) subjects versus aged matched controls we discovered a previously unidentified pool of the disease associated proteins in vesicles isolated from the frontal cortex of AD subjects.
Conclusion: Progression in understanding the role of extracellular vesicles in the nervous system has been hindered by a lack of appropriate methodology to isolate genuine exosomes, as defined by a minimal set of experimental requirements, from tissue. Our innovative methods have enabled us to isolate human brain exosomes and in doing so discover a new pool of neurodegenerative disease associated protein.

PT02.02

Isolation of exosomes from large volumes of cell culture media by ultrafiltration is superior to ultracentrifugation for the analysis of exosomal RNA

Csilla Terezia Nagy[1], Krisztina Pálóczi[2], Ágnes Kittel[3], Zsófia Onódi[1], Edit I Buzás[2], Péter Ferdinandy[1] and Zoltan Giricz[1]

[1]Department of Pharmacology, Semmelweis University, Budapest, Hungary; [2]Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary; [3]Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary

Introduction: Here we analysed protein and nucleic acid content of samples obtained from large volumes of cell culture supernatants by ultracentrifugation and different ultrafiltration methods to assess their applicability in downstream protein and nucleic acid analyses.
Methods: 3T3 fibroblasts and H9c2 cardiomyocytes were cultured in FBS-free DMEM-based medium for 24 h. Supernatants of 2.5 × 10^7 cells (200 mL) were centrifuged at 2500g, filtered on 0.8 μm PVDF membranes, centrifuged at 13,500g for 40 min. Supernatants were then either ultracentrifuged (UC) for 6 h at 100,000g or ultrafiltered on regenerated cellulose membranes with 100 kDa (UF100) or 10 kDa (UF10) cutoff rate. Filtrates from 100 kDa filters were ultrafiltered on 10 kDa cutoff rate filters (UF100 + 10). Protein content was measured by BCA method, then relative quantity of exosomal markers was assessed by western blot. Nucleic acids were studied by A260/280 method and capillary gel electrophoresis before and after DNase treatment. MicroRNA content was measured by PCR.
Results: Total protein concentration of UC, UF100, UF10 and UF100 + 10 samples were comparable. However, TSG101, Alix and Syntenin content of UC samples were higher than UF100 and UF10 samples. Exosomal protein content of UF100 + 10 samples was negligible. These results demonstrate that isolation of exosomes by 100 kDa filter is less efficient than UC and that 10 kDa filters retain more non-vesicular substances. UF100 samples contained more nucleic acid than UC samples. Gel electrophoresis and DNase treatment indicated that DNA contamination was the highest in UC samples, and that RNA content of UF100 samples were the highest, however, DNA contamination was significant in all samples. MicroRNA content of UF100 samples were the highest.
Conclusion: Although ultracentrifugation retains more exosomes than ultrafiltration, the latter method results in exosomal RNA of higher quantity and quality, therefore, more suitable for RNA analyses after DNase treatment.

PT02.03

Isolation of serum exosomes by optimised size-exclusion chromatography

Jik Han Jung and Ji Ho Park

KAIST, Daejeon, Republic of Korea

Introduction: Exosomes are natural nanoparticles ranging from 20 to 150 nm in size and having phospholipid bilayers. Recently, size-exclusion chromatography (SEC) have been studied as one of isolation methods for improving purity of isolated exosomes. However, SEC isolation of exosomes from phygiological sources such as serum still has been challenging in the aspect of purity because serum contains lipoproteins whose size is simillar to that of exosomes. Thus, we studied size distribution of exosomes and lipoproeins from cell supenatant and serum, and optimised SEC to improve the purity of isolated exosomes.
Methods: Luekemia cells (THP-1) were cultured for cell supenatant and human serum samples were kindly provided by "Korea University Anam Hospital". Column was packed with 10 ml of sepharose 2B and 6B resin to prepare SEC with different pore size. Then 0.5 ml of sample was loaded on the top of column, and each 0.5 ml eluate was collected. Each fraction of eluates was analysed by bicinchoninic acid (BCA) assay, dynamic lighting scattering (DLS), western blot, and transmission electron microscopy (TEM).
Results: In case of cell supenatant, exosomal marker CD63 was detected in fractions 9–11 and lipoprotein marker ApoB was mainly detected in fractions 10–13 with sepharose 2B column. Interestingly, In case of serum, CD63 was detected in fractions 11–15 and ApoB was still detected in fractions 9–13. To improve purity of isolated exosomes, serum was seperated by sepharose 6B column. As a result, CD63 was detected in fractions 12–14 and ApoB was detected in fractions 9–11.
Conclusion: In this work, we studied size distribution of exosomes and lipoproteins from cell supenatant and serum. We found that size distribution of lipoproteins was not dependent on sample type, and size of serum exosomes was smaller than that of exosomes from cell supenatant. We demonstrated that sepharose 6B is more suitable than sepharose 2B to isolate exosomes from serum.

PT02.04

The importance of isolation technique when analysing adipocyte markers in plasma-derived extracellular vesicles

Katherine D. Connolly[1], Rebecca M. Wadey[1], Aled Rees[2] and Philip James[1]

[1]Cardiff Metropolitan University, Cardiff, United Kingdom; [2]Cardiff University, Cardiff, United Kingdom

Introduction: Despite the known release of extracellular vesicles (EVs) from adipocytes, few reports exist detailing the presence of adipocyte-derived EVs in the circulation. One reason for this may be the lack of a distinct marker for adipocyte EVs, further complicated by the solubility of adipocyte-specific proteins such as adiponectin, fatty acid binding protein (FABP)-4 and peroxisome proliferator-activated receptor (PPAR)-γ2. We aimed to compare the detectability of adipocyte markers in plasma EVs isolated by differential ultracentrifugation and size exclusion chromatography.
Methods: Citrated blood was double-spun to yield platelet-poor plasma which was then either directly ultracentrifuged or loaded onto a size exclusion column to isolate plasma-derived EVs. Thirty fractions were collected from the column and analysed for protein content using Nanodrop and particle count using nanoparticle tracking analysis. Lysates of ultracentrifuged plasma EVs and pooled column fractions were compared by Western Blot for a series of hallmark adipocyte markers.
Results: Particle concentration, protein content and Western Blot analysis for markers indicative of an EV population, such CD9, identified fractions 5–10 as "EV rich". These fractions were pooled and ultracentrifuged in subsequent experiments. Adiponectin, FABP-4 and PPARγ2 were detected in both ultracentrifuged and column-derived EVs, however the signal was greatly reduced in column-derived EV fractions.
Conclusion: The soluble nature of many adipocyte-specific proteins poses difficulties when analysing a mixed population of EVs for adipocyte markers. Our results indicate that isolation of plasma-derived EVs by differential ultracentrifugation alone may result in contamination of the EV population with soluble adipocyte markers. Use of size exclusion chromatography columns followed by ultracentrifugation appears to separate EVs from the majority of soluble protein, thus reducing potential overestimations in adipocyte markers within plasma EVs isolates. Our data suggest that care must be taken when analysing plasma-derived EV fractions for adipocyte markers and the effects of the pre-isolation technique must be considered.

PT02.05

Filtration based method to deplete bovine extracellular vesicles from foetal bovine serum

Roman Kornilov[1], Maija Puhka[2], Hanna Hiidenmaa[1], Hilkka Peltoniemi[3], Bettina Mannerström[1], Riitta Seppänen-Kaijansinkko[1] and Sippy Kaur[1]

[1]Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, Finland; [2]Institute for Molecular Medicine Finland FIMM, University of Helsinki, Finland; [3]Laser Tilkka Ltd, Helsinki, Finland

Introduction: Foetal bovine serum (FBS) is the most common supplement used in cell culture experiments. Based on the recent evidence, FBS contain a large amount of extracellular vesicles (EVs) which hamper downstream analyses of secreted EVs. Therefore, it is important to eliminate EVs from FBS prior to cell culturing. Our primary aim in this study was to develop the cost-effective protocol to deplete the bovine EVs from FBS, and to analyse the effects of our EV depleted FBS on cell proliferation and metabolism.
Methods: EVs were measured from our EV-depleted FBS (ultra-15 centrifugal filters) and compared with commercially available (Shop) EV-depleted FBS and 19 hours ultracentrifuged (UC) EV-depleted FBS by nano tracking analysis, electron microscopy and Western blotting. The response of all three EV-depleted FBSs on cell proliferation and metabolism were measured on 3 different donors of mesenchymal stem cells.
Results: Efficiency of our FBS-EV elimination method was improved as compared with Shop EV-depleted FBS, and clearly better than 19 hours UC-FBS. Mesenchymal stem cells were grown in culture media using Shop-FBS, 19 hours UC-FBS and our EV-depleted FBS. Based on cell proliferation and metabolism analysis, all three EV-depleted FBSs maintained cell growth and metabolism up to 96 hours.
Conclusion: Our results indicate that our protocol shows efficient depletion of EVs, is cost effective, easy to use and maintains the cell growth and metabolism of mesenchymal stem cells *in vitro*.
Roman Kornilov and Sippy Kaur are having equal contribution.

PT02.06

Increasing the isolation yield of EVs from oral cancer cells in culture

Eduarda M. Guerreiro[1], Anne-Marie Trøseid[2], Reidun Øvstebø[2], Tine M. Søland[1] and Hilde Galtung[1]

[1]Department of Oral Biology, Faculty of Dentistry, University of Oslo, Norway; [2]The Blood Cell Research Group, Department of Medical Biochemistry, Oslo University Hospital, Ullevål, Norway

Introduction: To get a high yield of extracellular vesicles (EVs) from cell culture experimental set-ups, classic cell culture methods require a high number of flasks, which is a practical and economic burden. A promising approach was found in the work by Mitchell and colleagues (1) using the Integra CELLine culture system (Integra Biosciences AG, CH). The use of this semi-continuous, three-dimensional culture system allows a high cell density, that yielded an increase in isolated EVs. Therefore, the aim of this study was to test and determine if the Integra CELLine system is a better alternative to increase the yield of EVs from an oral squamous cell carcinoma (OSCC) cell line compared to traditional flasks.
Methods: PE/CA-PJ49 (OSCC) cells were cultured in Advanced DMEM (Gibco) with L-glutamine, PSA, and exosome-depleted FBS (1% V/V, Thermo Fisher Scientific) in T-175 flasks for 96 h and in the Integra CELLine system for 8 days. Conditioned culture media were collected and concentrated by ultrafiltration (Amicon Ultra centrifugal filters, Ultracel 50 K – 50 kDa cut-off) and loaded into size exclusion chromatography columns (Sepharose CL-2B, GE Healthcare). Particle number in the different fractions was determined by nanoparticle tracking analysis (NTA) using Nanosight NS500, followed by western blot using anti-CD9 antibody.
Results: There was a sixfold increase in the total amount of vesicles in the Integra CELLine system cell culture supernatant when compared with the T-175 supernatant, as shown by preliminary results from NTA. We also documented, by western blot, increased CD9-levels in the samples from the Integra CELLine supernatant in comparison to that of the T-175 flasks.
Conclusion: Our results support that the use of the Integra CELLine system is a promising approach to increase the yield of EVs from oral cancer cells in culture. More details on the recovered EVs are to be elucidated by magnetic bead-bound CD9 positive EVs by flow cytometry, electron microscopy and western blot analysis of other markers.

References

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PT02.07

Exosome isolation with carbonate treatment from cell culture supernatant and human serum

Lifang Yang, Vanessa L. Correll, Jamie L. Eisner, Cristinia M. Risi, Vitold E. Galkin and Oliver J. Semmes

Eastern Virginia Medical School, VA, USA

Introduction: Exosomes are small vesicles which have been implicated as potential vehicles of targeted drug delivery and important reservoirs of novel disease biomarkers. The current "gold-standard" for exosome purification is

ultracentrifugation (UC)-based method, which typically involves an additional clean-up step by applying PBS wash between two UCs. However, this method is known to suffer from non-vesicular macromolecule contamination. To this end, we integrated alkaline carbonate treatment (pH = 11.0) into UC-based protocol and optimised it for exosome isolation. We tested the utility for the efficiency, yield, purity and function of isolated exosomes both from cell culture supernatants and complex biological body fluids such as serum.
Methods: Exosomes were isolated from the conditioned media from prostate cancer DU145 cells and human normal serum using new protocol (UC-Alk) and compared with those using traditional UC-based protocol (UC-PBS). The concentration and size distribution of vesicles were analysed by nanoparticle tracking analysis (NTA). The morphology was visualised by transmission electron microscopy (TEM). The floating density was measured in a linear sucrose density gradient. The specificity was evaluated by western blot and flow cytometry. The function was assessed by uptake of labelled DU145-derived exosomes by prostate stromal WPMY-1 cells.
Results: Both isolations from DU145 supernatants contained 50–150 nm vesicles, were positive for canonical exosome markers (Alix, TSG101, syntenin-1, CD9, CD63) and absent for intracellular organelles. However, UC-Alk outperforms UC-PBS in terms of purity as illustrated by the cleaner nanovesicles on TEM, the higher enrichment in exosomal membrane proteins, and the narrower buoyant density (1.11–1.16 g/ml). When the new method was applied for human serum, UC-Alk demonstrated significantly lower background and enhanced exosome signal devoid of highly abundant serum proteins. In the context of cellular uptake, the exosomes isolated by UC-Alk were internalised by target cells indicating that they were not damaged by alkaline wash and indeed biologically active.
Conclusion: Our optimised exosome isolation strategy is a valuable tool to investigate exosome-specific functions and clinical applications.

PT02.08

Purification method affects biological functionality of stem cell-derived EVs

Sander A.A. Kooijmans[1], Sara Previdi[2], Daniel Moya Rull[3], Sharad Kholia[1], Pieter Vader[2], Raymond M. Schiffelers[4] and Giovanni Camussi[5]

[1]Bioindustry Park Silvano Fumero SpA; [2]University Medical Centre Utrecht, Utrecht, The Netherlands; [3]Laboratori Experimental de Nefrologia i Trasplantament (LENIT); [4]Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, Utrecht, The Netherlands; [5]University of Turin, Department of Medical Science, Torino, Italy

Introduction: Extracellular vesicles (EVs) from a variety of stem cells are believed to harness regenerative capacity, which may be exploited for therapeutic purposes. For example, EVs from human liver stem cells (HLSCs) and mesenchymal stem cells (MSCs) have been shown to stimulate regeneration of damaged kidney tissue. However, EV activity may depend on the employed purification method, which limits cross-study comparisons. Here, we investigated the effect of the purification method on *in vitro* regenerative effects of HLSC- and MSC-derived EVs.
Methods: Human proximal tubule cells (HK2) were exposed to HLSC- and MSC-derived EVs, which were purified using a standard ultracentrifugation (UC), ultracentrifugation + wash (UCW) or size-exclusion chromatography (SEC) protocol. EVs were quantified and characterised by NTA, protein assays and bead-based flow cytometry. HK2 proliferation was determined using BrdU proliferation assays.
Results: EV particle yield was generally similar among purification methods, although EV purity (defined as particle/protein ratio) was different, and decreased in the order SEC > UCW > UC. EV purity markedly correlated with their ability to stimulate proliferation of HK2 cells. Importantly, "non-EV" fractions from SEC purifications also showed biological activity in this readout assay.
Conclusion: Our data show that purification methods (and resulting EV purity) greatly influence regenerative effects of stem cell-derived EVs in *in vitro* readout assays. This may lead to data misinterpretation and thereby hamper therapeutic development. Hence, the presence and quantity of EV contaminants should be considered when assessing biological activities of EVs.

PT02.09

Influence of commercially available, exosomal isolation kits on holistic small RNA expression profiles of serum in healthy and critically ill individuals

Benedikt Kirchner[1], Dominik Buschmann[1], Stefan Kotschote[2], Michael Bonin[2], Marlene Reithmair[3], Gustav Schelling[4] and Michael Pfaffl[1]

[1]Division of Animal Physiology and Immunology, TUM School of Life Sciences Weihenstephan, Technical University Munich, Germany; [2]IMGM Laboratories GmbH; [3]Institute of Human Genetics, University Hospital, of Ludwig-Maximilians-University Munich, Germany; [4]Department of Anaesthesiology, University Hospital, Ludwig-Maximilians-University, Munich, Germany

Introduction: Due to the unique role that extracellular vesicles (EVs) and their cargo play in cell-to-cell communications of a multitude of physio- and pathophysiological conditions, exosomes have become an important object of research especially in biomarker development. A number of kits have emerged on the market, taking advantage of various biochemical and physical properties to isolate exosomes from biofluids or cell-culture supernatant. Unfortunately a thorough comparison of the different isolation strategies (e.g. membrane affinity, precipitation, size exclusion chromatography), especially in the context of clinically relevant settings or samples like liquid biopsies, is still missing.
Methods: EVs were isolated from 1 ml serum of healthy individuals and critically ill patients (n = 10 each) using four different commercially available isolation kit alongside differential ultra-centrifugation (n = 8). Total RNA yield and integrity were evaluated using capillary gel electrophoresis and holistic small RNA expression profiles were generated by NGS. EV isolation kit-specific influences were assessed by comparing library size, sequence length distribution, unsupervised clustering and differential expression analysis between sample matrices as well as isolation strategies.
Results: Total RNA yield differed greatly (p = 0.002) between isolation strategies with precipitation (4505 ± 3329 pg/µl) greatly outperforming size-exclusion chromatography (157 ± 197 pg/µl). Sampling from critically ill patients reduced RNA yield for all methods by a factor of 1.5–3.8 (p = 0.002). Even more striking differences were revealed by small RNA NGS. Although all isolation strategies were able to distinguish between samples from healthy and critically ill individuals to a certain degree, mapped miRNA expression profiles varied significantly.
Conclusion: A major impact on small RNA expression profiles could be shown for all EV isolation kits and strategies, respectively. Our findings highlight the importance of further optimisation and standardisation of exosomal isolation methods in differing sample matrices and special attention needs to be paid to obtain reproducible and comparable biomarker signatures from liquid biopsies.

PT02.10

Assessing cell culture parameters for enhanced bioactive extracellular vesicle production

Divya Patel[1], Kelsey Gray[2], Yasasvhinie Santharam[2], Kim Stroka[2] and Steven M. Jay[1]

[1]University of Maryland, College Park, MD, USA; [2]University of Maryland, MD, USA

Introduction: Although extracellular vesicles (EVs) derived from bone marrow derived mesenchymal stem cells (MSCs) and other cell types are implicated in promoting vascularisation, their clinical translation is limited by the lack of a large-scale biomanufacturing approach. Increased understanding of how cell culture parameters such as cell passage and cell seeding density influence EV biogenesis and bioactivity has the potential to enhance therapeutic EV production. Here, we investigate the impact of these parameters on MSC-derived EV production and vascularisation bioactivity.
Methods: Conditioned media was collected after 24 h from MSCs seeded at different densities (1E2, 5E2, 1E3, 1E4 cells/cm^2) or passages (P2-P5). EVs were isolated from the conditioned media via differential centrifugation and quantified by nanoparticle tracking analysis (NTA) using a

Nanosight LM10 and CD63 ExoELISA. Vascularisation bioactivity of isolated EVs was assessed in a wound healing assay.
Results: NTA and ExoELISA results indicated increased EV production rates per cell when MSCs were seeded at lower initial densities, regardless of the cell passage. The average fold decrease in EVs production per cell between cells seeded at 1E2 cells/cm^2 and 1E4 cells/cm^2 for P2, P3, P4, and P5 was 100, 85, 110, and 50, respectively (n = 5, $p < 0.01$). Additionally, multiple EV collection time points (12 and 24 h) from the same cells increased total EV production more than 3 fold compared to a single collection over the same time period (24 h) (n = 3, $p < 0.05$). Seeding density had no affect on the vascularisation bioactivity of MSC EVs produced as assessed by the wound-healing assay (n = 3). In contrast, increasing cell passage was correlated with diminished EV bioactivity (n = 3).
Conclusion: These results suggest that high EV production rates can be achieved by seeding cells at lower initial seeding densities. Low cell passage number is critical to retaining MSC EV vascularisation bioactivity. The implications of these findings are that higher amounts of bioactive EVs can be achieved using a lower number of producer cells with increased frequency of collection. This may allow for significant reduction in cost of EV production and begin to inform the rational design of a large-scale biomanufacturing approach for therapeutic EV production.

PT02.11

Evaluation and optimisation of a hollow fibre bioreactor system for standardisation of large scale production of extracellular vesicles

Ulrika Felldin[1], Giulia Corso[1], Bernd Giebel[1,2], Helmut Hanenberg[3], Joel Z. Nordin[1], Samir El-Andaloussi[1,4] and André Görgens[1,2]

[1]Department of Laboratory Medicine, Karolinska Instiutet, Stockholm, Sweden; [2]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; [3]Department of Pediatrics III, University Children's Hospital Essen, University of Duisburg-Essen, Essen, Germany; [4]Department of Physiology, Anatomy and Genetics, University of Oxford, United Kingdom

Almost all types of cells release extracellular vesicles (EVs) which are involved in a plethora of both physiological and pathological processes. EVs have inecent years been connected to various therapeutic approaches including anti-tumour therapy, vaccination, modulation of the immune system and drug-delivery. Translating exosome-based therapies to the clinic, however, requires a large-scale production of exosomes, and a subsequent comprehensive evaluation, optimisation and standardisation of all parameters during production. Bioreactors are regularly used to grow cells in 3D-matrices at high densities, which may be more similar to native *in vivo* conditions than classical 2D cultures.
Aiming to scale-up EV production, we are setting up and evaluating a commercially available hollow fibre bioreactor system with 20 kDa molecular weight cut off pores. So far, we started to culture different cell types, including a stable HEK293T-CD63eGFP cell line that secretes eGFP-positive EVs. Cell proliferation kinetics within bioreactors are monitored by glucose uptake, and the production of EVs both under serum-supplemented and defined serum-free conditions is currently evaluated. The concentration and size distribution are measured by nanoparticle tracking analysis (NTA) and surface marker expression profiles and uptake kinetics in recipient cells of harvested EVs are analysed via flow cytometry. All parameters are compared to classical 2D culture. Also, different schedules for EV harvesting are compared in order to optimise and standardise the production. Preliminary results and experiences using hollow fibre bioreactors for the large-scale production of EVs from different cell types will be presented here.

PT02.12

Purifying and molecular profiling extracellular vesicles (EVs) from various biological specimens

Abiodun Ogunjimi[1] and Liang Zhang[2]

[1]Lunenfeld-Tanenbaum Research Institute; [2]City University of Hong Kong, China

Introduction: It is known that all cell types release extracellular vesicles (EVs), which are membrane vesicles with sizes in the nanometre to micrometre range. EVs carry a broad spectrum of bioactive molecules including proteins, lipids, RNA, DNA, etc, which may specifically reflect not only the identity, but also the physiological and pathological status of the source cells. Therefore intense research efforts are undergoing to characterise the molecular profiles and mechanisms of EVs-mediated cellular communications in healthy and disease conditions. Such efforts have the potential to identify EVs-based biomarkers and/or therapeutic targets for various diseases. Advances in isolating and profiling technologies have greatly improved our knowledge of EVs in various biological specimens. However, biological specimens including serum, urine, spinal fluid, semen, etc. display huge variations in available volumes, as well as their biophysical and biochemical properties, such as viscosity and protein concentration. Currently, a major limitation in the field of EVs study is the lack of standardisation for isolating and profiling of EVs from different specimens.
Methods: We compared major isolation methods in the field for their efficiency in purifying EVs from cell-culture conditional media, serum and urine. The isolated EVs are subjected to proteomic and RNA analysis to evaluate the effects of different isolating strategies on the results of molecular profiling.
Results: Depending on the nature of biological specimens and available volumes, different isolating methods display large variations in the efficiency of EVs purification. Interestingly, molecular profiling of the EVs from the same biological specimen also vary significantly among different isolating methods.
Conclusion: Our studies indicate that it is preferable to use distinct isolating method for different biological specimens and that optimised workflow is key to obtaining reliable molecular profiling of EVs.

Poster Session PT03 – EVs in Tissue Protection and Repair
Chairs: Uta Erdbruegger and TBD 5:15–6:30 p.m.

PT03.01

Protective role of extracellular vesicles in diabetic microangiopathy

Chiara Gai, Tatiana Lopatina, Yonathan Gomez, Maria Felice Brizzi and Giovanni Camussi

Department of Medical Sciences, University of Turin, Torino, Italy

Please see OPT02.01

PT03.02

Significant improvement of survival of rats with acute liver failure by high concentration exosome of human adipose-derived stem cells

Yinpeng Jin, Hongchao Li, Junyi Wang, Lingyu Meng, Li Li, Xiaojin Wang, Chengwei Chen and Qingchun Fu

Shanghai Liver Disease Research Centre,The 85th Hospital of PLA

Please see OPT02.02

PT03.03

Exosomes derived from GATA-4 overexpressing mesenchymal stem cells rejuvenate cardiomyocytes through transfer miRNAs to regulate the related signalling pathway

Bin Yu[1], Min Gong[1], Yigang Wang[1], Muhammad Ashraf[2] and Meifeng Xu[1]

[1]University of Cincinnati, OH, USA; [2]University of Illnois in Chicago, IL, USA

Our previous studies indicate that mesenchymal stem cells (MSC) which overexpress GATA-4 (MSC^{GATA-4}) are capable of reducing infarction size of ischemic myocardium and promoting cardiac function recovery. Here, we investigated whether exosomes (EXO) released from MSC^{GATA-4} rejuvenate cardiomyocytes (CMs) as evidenced by reducing CM apoptosis and senescence, enhancing proliferative capacity of CM through transferring miRs and regulating related signalling pathways.

EXO were isolated from rat bone marrow MSC transduced with GATA-4 or with its vector-empty control. Mature CMs were harvested from adult Sprague-Dawley rat ventricles. EXO significantly increased CM survival, reduced cell damage caused by exposure to hypoxia for 48 h in a concentration-dependent manner and the action was enhanced in EXO obtained from MSC^{GATA-4} (Exo^{GATA-4}). CMs were cultured in serum free medium containing either EXO or 1% BSA for 3 weeks. Exo^{GATA-4} significantly reduced the number of senescence-associated β-galactosidase positive CMs and restored their beating frequency. The dedifferentiation and proliferation of mature CMs was recorded by a real-time imaging system. The cell number was significantly greater in CMs cultured in the serum-free medium contained EXO than those treated with BSA. The percentages of $Ki67^+$ CMs and EdU^+ CMs were significantly higher in the group treated with Exo^{GATA-4} compared to that of the groups treated with BSA and EXO obtained from control MSC (Exo^{null}). Furthermore, the data of MicroRNA-seq showed that 358 miRs were found in EXO and 44 miRs were significantly increased in Exo^{GATA-4} compared to Exo^{null}, including let-7 family members. Addition of EXO pre-labelled with PKH26 into CM cultures showed that EXO were quickly internalised by CMs and the expression of let-7 miRs in CMs was significantly upregulated. PTEN, one target of let-7 and several enriched miRs in Exo^{GATA-4}, was significantly down-regulated in CMs treated with Exo^{GATA-4}. In contrary, loss-function experiments showed that down-regulation of let-7 in Exo^{GATA-4} significantly abrogated the therapeutic effect of Exo^{GATA-4}.

All these data suggested that Exo^{GATA-4} rejuvenated CM and promoted mature CM cell-cycle re-entry and proliferation through delivering miRs and regulating signalling pathways.

PT03.04

Evaluation of the contribution of extracellular vesicles secreted by multipotent mesenchymal stromal cells in MSC-mediated regenerative effects

Georgy Sagaradze[1], Anastasia Efimenko[2], Liudmila Ageeva[1], Natalia Kalinina[1], Natalia Basalova[3], Pyotr Nimiritskiy[1], Anna Vnukova[4], Evgeniy Evtushenko[5], Olga Makarevich[1] and Vsevolod Tkachuk[1,2]

[1]Department of Biochemistry and Molecular Medicine, Lomonosov Moscow State University, Moscow, Russia; [2]Institute of Regenerative Medicine, Lomonosov Moscow State University, Moscow, Russia; [3]Department of Cytology and Histology, Lomonosov Moscow State University, Moscow, Russia; [4]I.M. Sechenov's First Moscow State Medical University, Moscow, Russia; [5]Department of Chemical Enzymology, Lomonosov Moscow State University, Moscow, Russia

Introduction: Adipose-derived mesenchymal stem/stromal cells (MSC) represent a promising source of stem and progenitor cells for regenerative medicine. MSC were shown to support regeneration and reparation in various experimental conditions and clinical trials. MSC function by secreting growth factors, cytokines, extracellular matrix proteins, as well as extracellular vesicles (EV). Thus, conditioned medium (CM) containing cell-secreted components stimulate regenerative processes comparable with MSC themselves in many clinical models. By present data, EV are considered to be the most potent components in MSC secretome. EV carry a set of proteins, bioactive lipids, nucleic acids, protected by a lipid bilayer, and demonstrate persistent regenerative effects, when absorbed by target cells. However, many investigators show, that CM components, other than EV, also participate in MSC function. Thus, to clear the mechanisms of MSC regenerative effects it is important to estimate contribution of EV in these processes.

Methods: We separated EV and soluble components of MSC CM using the ultracentrifugation. To visualise EV and to identify major EV markers we performed transmission electron microscopy and western blotting, respectively. We estimated effects of EV in angiogenesis, neuritogenesis, and wound healing models *in vitro*.

Results: We found that impact of EV in the stimulation of endothelial cell capillary-like structure formation and neuroblastoma cell line neuritogenesis was substantial. In contrast, EV less stimulated functions of dermal fibroblasts in wound healing models. We also enriched EV fraction with distinct EV subtypes using chemical inhibitors to analyse the impact of these subtypes in MSC effects.

Conclusion: Identity of the most potent components secreted by MSC, particularly EV subtypes, and selection of distinct conditioned medium fractions affecting different cell types will allow to produce more efficient therapeutic formulations for stimulation of regeneration and reparation in the future.

PT03.05

Neural stem cell-derived exosomes protect the enteric nervous system and promote intestinal motility after necrotising enterocolitis

Yu Zhou[1], Chris McCulloh[2], Jacob Olson[2] and Gail Besner[2]

[1]Department of Pediatric Surgery, Nationwide Children's Hospital; [2]Nationwide Childen's Hospital

Introduction: Necrotising enterocolitis (NEC) is the most common cause of gastrointestinal-related mortality in premature babies. We have shown that neural stem cell (NSC) transplantation protects the enteric nervous system (ENS) during experimental NEC, but it is unclear whether SC engraftment or SC-secreted products mediate these effects. SC-secreted exosomes are cell-

derived nanosized microvesicles that are involved in mediating intercellular communication. The aim of this study was to test the effects of SC-derived exosomes in animals subjected to experimental NEC.
Methods: Enteric NSC were isolated from neonatal rat intestine, neurosphere-like bodies cultured, and NSC-secreted exosomes isolated from the condition medium. Exosomes were labelled with PKH26 red dye and delivered to intestinal neurons subjected to anoxia/reoxgenation (A/R) injury. Neuronal apoptosis was determined by caspase 3 immunohistochemistry and flow cytometry using Annexin V. *In vivo*, fluorescently labelled exosomes were administered intraperitoneally (IP) to rat pups exposed to experimental NEC. Intestinal exosome distribution was examined using a Xenon imaging system. Intestinal injury was graded histologically and the incidence of NEC determined. Intestinal motility was examined in intestinal segments mounted in an organ bath using electrical field stimulated (EFS) electromyography.
Results: NSC-derived exosomes specifically targeted injured neurons *in vitro*, and significantly decreased A/R-induced neuronal apoptosis (36.7% ± 3.2% *vs*. 6.49% ± 2.4% ratio of apoptotic neurons/total neurons, $p < 0.05$). *In vivo*, exosomes administered IP homed to injured intestinal myenteric neurons in pups exposed to NEC, leading to decreased intestinal histologic injury and significantly decreased mortality (71% *vs*. 40%, $p < 0.05$). NEC-induced impairment in EFS isometric contractility was restored by NSC-derived exosome administration.
Conclusion: NSC-derived exosomes protect intestinal neurons from injury *in vitro*, and protect the intestines from NEC *in vivo*, suggesting that they mediate the therapeutic efficacy of NSC. NSC-derived exosomes may represent a novel non-cell based therapy for improving the recovery of neuromuscular function and protecting the ENS from NEC.

PT03.06

Exosomes derived from human mesenchymal stem cell accelerate wound healing in a mouse model of radiation-induced injury

Alexandre Ribault[1], Stephane Flamant[1] and Radia Tamarat[2]

[1]IRSN/PRP-HOM/SRBE/LR2I; [2]IRSN/PRP-HOM/SRBE

Introduction: Mesenchymal stem cells (MSCs) are multipotent cells which have been reported to promote the regeneration of skeletal muscle and skin wound healing in pre-clinical animal studies. MSCs derived exosomes, containing diverse proteins, mRNAs and micro-RNAs, mediating various biological functions, might be a main paracrine mechanism for stem cell to mediate their therapeutic effect. Recent studies have shown that exosomes derived from MSCs have regenerative functions in several tissues, including skin, skeletal muscle, kidney and heart. We hypothesised that exosomes could participate to the wound closure of radiological burns in mice.
Methods: Mice were irradiated with X-ray at 80 Gy. 14 d after irradiation, PBS or Adipose derived MSCs (10^6 per animal) or exosomes (400 µg or 800 µg) were injected all around the injury. Exosomes were obtained from culture of human embryonnic stem cells-derived c-myc-immortalised MSCs. Every 7 d after irradiation mice were scored according to the the limb retraction, the wound extent, inflammation and humidity. 28 d after irradiation mice were euthanised and the skin and muscle of the irradiated limb were recolted for further analyses.
Results: From 7 d after injections we observed that animals injected with 800 µg of exosomes tended to have a similar wound compared to the day of injection while animals injected with PBS, MSCs and 400 µg of exosomes showed a bigger one. 14 d after injections, the trend was confirmed and animals injected with 800 µg had the smaller scoring (81[±9]) compared to the other groups (88[±8], 90[±10] and 93[±9] for PBS, MSC and 400 µg of exosomes groups respectively).
Conclusion: Our preliminary data seem to show that MSCs-derived exosomes had a beneficial effect on the wound closure of radiation induced-injuries in a dose effect manner. 400 µg of exosomes are not sufficient to promote an efficient wound healing while 800 µg are. Further analyses are necessary to determine what parameters are influenced by exosomes.

PT03.07

Human amnion epithelial cells release vesicles that rescue bleomycin induced lung injury in mice

Jean Tan[1], Sinnee Lau[1], Euan Wallace[2], Lois Salamonsen[1], Hong Nguyen[3], Dandan Zhu[4], Carla Kim[5] and Rebecca Lim[1]

[1]Hudson Institute of Medical Research, Clayton, Australia; [2]Monash University, Clayton, Australia; [3]Melbourne University, Melbourne, Australia; [4]Postdoc; [5]Harvard University, MA, USA

Introduction: The human amnion epithelial cells (hAECs) release extracellular vesicles (EVs) that appear to reflect the pro-reparative properties of the hAECs themselves. A proteomics screen confirmed that hAEC-EVs package immunomodulatory molecules such as HLA-G, and ligands associated with stem cell niche maintenance, such as Wnt5A. RNA-Seq analysis revealed that eight miRNAs with associated anti-fibrotic effects were amongst the most abundant transcripts.
Aim: Given that lung fibrosis is though to be perpetuated by stem cell attrition, we sought to evaluate the potential of hAEC-EVs to reverse lung fibrosis in aged mice.
Methods: Eleven-to-twelve month old female C57Bl6 mice ($n = 6$ per group) were challenged with bleomycin (0.15 U/kg) and fibrosis allowed to develop. On the 14th day post challenge, either 10 mg EVs resuspended in saline, or vehicle alone, was instilled intranasally. Mice were culled 28 days post challenge. Tissues were collected for histological analysis and endogenous lung stem cells (bronchioalveolar stem cells, CD31-, CD45-, EpCAM+, Sca1hi) were flow sorted for gene expression analysis using the Fluidigm Biomark HD.
Results: The hAEC-EVs were well-tolerated, with no morbidity or mortality associated with the intervention. Indeed, the hAEC-EVs reversed fibrosis to levels comparable to healthy controls and this was associated with a 50% reduction in myofibroblast activation.
Conclusion: hAEC-EVs exert potent anti-fibrotic effects when delivered intranasally to aged mice challenged with bleomycin. This appears to be associated with their ability to either activate endogenous stem cells within the lung or protect the stem cell niche during the injurious process.

PT03.08

Biological properties and regenerative potential of murine bone marrow mesenchymal stem cell-derived extracellular vesicles in heart repair

Anna Labedz-Maslowska[1], Guangming Cheng[2], Malgorzata Sekula[3], Yu-Ting Xuan[2], Elzbieta Karnas[3], Sylwia Kedracka-Krok[3], Robert Vincent[2], Michal Sarna[3], Zbigniew Madeja[4], Buddhadeb Dawn[2] and Ewa K. Zuba-Surma[1]

[1]Department of Cell Biology, Jagiellonian University, Krakow, Poland; [2]Cardiovascular Research Institute, University of Kansas Medical Center, Kansas City, KS, USA; [3]Malopolska Centre of Biotechnology, Jagiellonian University, Krakow, Poland; [4]Jagiellonian University, Krakow, Poland

Extracellular vesicles (EVs) represent membrane-enclosed vesicles released by normal and activated cells including stem cells (SC) playing a role in cell-to-cell communication. Although, growing evidence indicates that mesenchymal SC (MSC)- derived EVs may play a pivotal role in several organ repair, their role in heart regeneration has not been well studied.
Thus, the aim of this study was to examine the bioactive content and regenerative capacity of murine bone marrow MSC- derived EVs in murine model of acute myocardial infarction (AMI) *in vivo*.
MSC-EVs were isolated from conditioned media via sequential centrifugation including ultracentrifugation at 100,000*g*. The morphological analysis of the 100 K EV fraction (including ectosomes and exosoms) by Izon system, revealed the presence of vesicles in average size about 200 nm. The vesicular morphology was confirmed by atomic force microscopy, while the protein markers were assessed accordingly to ISEV recommendations by western blotting. High-sensitivity flow cytometry (Apogee Flow system) confirmed the presence of several MSC-specific markers on MSC-EVs including receptors and adhesions. We also found MSC-EVs to be enriched in mRNAs, miRNAs and several

proteins from donor MSC cells as shown by real-time RT-PCR and mass spectroscopy, respectively. We found MSC-MVs to carry several transcripts regulating SC cardiac and angiogenic differentiation capacity. Importantly, our data (i) indicated a great impact of MSC-EVs on proangiogenic capacity of heart endothelial cells *in vitro* as well as (ii) confirmed their regenerative potential *in vivo* by showing improved heart histology, anatomy and function in murine AMI model. The increase in number of new capillaries in the place of EV injection, may suggest the increased perfusion as one of the major mechanisms involved in the MSC-EV regeneration capacity in *vivo*.
In summary, our data demonstrated that MSC-derived EVs represent natural nanocarriers transferring bioactive content to mature target cells and playing an effective role in heart regeneration *in vivo*.
We conclude that MSC-EVs may represent novel safe therapeutic tool in heart tissue regeneration, alternative or supporting to whole cell-based therapy in heart repair.

PT03.09

Biodistribution and efficacy of extracellular vesicles from cardiosphere-derived cells

Jennifer L. Johnson[1], Ahmed Ibrahim[1], Chris Sakoda[1], Kenny Gouin[2], Kiel Peck[1], Liang Li[1], Travis Antes[3], Houman Hemmati[1], Rachel Smith[1], Linda Marban[1] and Luis Rodriguez-Borlado[1]

[1]Capricor Therapeutics; [2]Cedars Sinai, CA, USA; [3]Cedars-Sinai Medical Centre, Heart Institute, CA, USA

Introduction: Extracellular vesicles produced by cardiosphere-derived cells (CDC-EVs) have been shown to recapitulate the therapeutic activity of parent cells in heart-related diseases. The ability of CDC-EVs to reduce inflammation, attenuate fibrosis, and activate regeneration make them very attractive for inflammatory diseases treatment. Capricor is evaluating the use of CDC-EVs for the treatment of ocular graft versus host disease (oGVHD), an indication where the product can be locally delivered. No previous studies have been published analysing EVs biodistribution after eye delivery. Here, we show *in vivo* biodistribution of CDC-EVs in an ocular alkali burn mouse model after subconjunctival or topical delivery, using a novel qPCR-based method. We also analysed the therapeutic potential of CDC-EVs in mouse and rabbit models. Finally, CDC-EVs uptake by different cellular types was analysed *in vitro* to identify CDC-EVs target cells.
Methods: Unmodified human CDC-EVs were injected into the subconjunctival space or administered topically to healthy or injured mouse eyes. *In vitro* uptake of dye-labelled EVs was measured by detecting intracellular fluorescence in treated cells by flow cytometry. *In vivo* biodistribution tracking was then performed using a sensitive qPCR method tracking a YRNA fragment abundant in CDC-EVs. Therapeutic activity of CDC-EVs was evaluated in a rat model of corneal alkali burn injury and a rabbit model of Sjögren's syndrome.
Results: Biodistribution studies showed a higher presence of EV-CDCs in the eyes after subconjunctival injection when compared to topical delivery. Retention of CDC-EVs in the eye was stronger in the presence of ocular damage. Biodistribution of CDC-EVs was different when compared to MSC-EVs. Efficacy studies showed that CDC-EVs have the potential to repair injured eye tissue in chronic and acute models. Finally, uptake studies revealed variable CDC-EVs uptake kinetics across different cell types following incubation.
Conclusion: Here we show uptake of human CDC-EVs by several cell types, reliable measurement of biodistribution after *in vivo* delivery using two administration routes, and efficacy of CDC-EVs in two disease animal models. These findings are critical in the advancement of EV therapy to clinical applications.

PT03.10

Hepatocyte-secreted extracellular vesicles modify endothelial function by an arginase-dependent mechanism

Félix Royo[1], Laura Moreno[2], Justyna Mleczko[3], Laura Palomo[1], Esperanza Gonzalez[1], Angel Cogolludo[2], Francisco Vizcaíno-Perez[2], Sebastiaan van Liempd[1] and Juan M. Falcón-Pérez[1]

[1]CIC bioGUNE; [2]Universidad Complutense Madrid, Madrid, Spain; [3]CIC bioGUNE-Liverpool University, Liverpool, United Kingdom

Introduction: Hepatocytes release extracellular vesicles (EVs) loaded with signalling molecules and enzymes into the bloodstream. Although the importance of EVs in the intercellular communication is already recognised, the metabolic impact of the enzymes carried by these vesicles is still unclear.
Methods: We isolated EVs secreted by primary rat hepatocytes by differential ultracentrifugation, and we evaluated the metabolic effect of these vesicles by performing untargeted metabolomic profiling of serum samples exposed to them. Afterwards, by using sucrose density gradients, biochemical and molecular analysis in *in vitro* and *in vivo* models we validated that some of the observed metabolic effects are caused by the arginase activity that is associated to small EVs. Finally, by using *ex vivo* pulmonary arteries we measured the effect of these arginase-carrying vesicles on the vascular function.
Results: We found significant changes in the abundance of 94 serum metabolic signals of different chemical nature including metabolites related to arginine metabolism, which regulates vascular function. We demonstrated the presence of arginase-1 protein and its activity in the hepatic EVs carrying the exosomal markers CD81 and CD63. Remarkably, the arginase activity was also detected in EVs isolated from the serum *in vivo*, and this vesicular activity significantly increased under liver-damaging conditions. Finally, we demonstrated that EVs secreted by hepatocytes inhibited the acetylcholine-induced relaxation in isolated pulmonary arteries, *via* an arginase-dependent mechanism.
Conclusion: The study demonstrates that hepatocytes secrete small EVs into the extracellular environment that are metabolically active and modify the levels of blood metabolites associated with energy and redox metabolisms, and endothelial regulation. Importantly, they are involved in an arginase dependent mechanism regulating the endothelial function locally and, possibly, at distant locations. This phenomenon could be relevant and have pathological implications for hepatopulmonary syndrome.

T03.11

Characterization of extracellular vesicles from different tumor cell lines

Corinna Plattfaut, Annika Freund, Tabea Quecke and Frank Gieseler

University of Luebeck, Luebeck, Germany

Introduction: Extracellular vesicles (EVs) are released by various cell types and can be found in body fluids. They contain biological material such as DNA and mRNA, they are able to activate the coagulation system and induce cellular signaling pathways through their membrane surface components. The amount of EVs in blood samples from patients has been correlated to inflammation and tumor activity; nevertheless, the clinical relevance is unclear. Here we show that cells from various tumors release EVs in substantial amounts and, that the release can be increased by cytokines. EVs were then characterized structurally and functionally.
Methods: Cell lines (all ATCC, human): OVCAR3, ovary adeno; Colo357, pancreas adeno; A549, lung epithelial; CaCo2, colon adeno; breast, epithelial. Incubation w/wo cytokines (TNF-alpha, TGF-beta). EVs were isolated by sequential centrifugation steps including high-speed (10.000 x g) as well as by capturing PS-presenting EVs by annexin-coated magnetic beads. Counting, characterization by Novocyte flow cytometer (488 nm laser) and MP-activity assay (PS presentation). Functional assays: MPTF-activity assay (TENase activity), ERK phosphorylation and tumor cell migration (Oris).
Results: We were able to isolate EVs from all tumor cell lines; EV release was doubled by stimulation with TNF alpha (inflammation). EVs were isolated by high-speed centrifugation or by capturing using annexin-coated magnetic beads. Although the amount of EVs released by the different tumor cells was comparable (MD 0.05), they differed significantly as well in the amounts of PS presented on the surface (MD 1.88), as their TENase activity (MD 0.50). Tumor cell EVs induced ERK phosphorylation and some induced tumor cell migration. Inhibition of ERK phosphorylation as well as PAR2 inhibition reduced tumor cell

migration notably, which points to the involvement of PAR2 - small G proteins - ERK signaling pathway.
Conclusion: These observations indicate that clinical effects such as the activation of coagulation or tumor cells are not only related to the amount of EVs; structural and functional characteristics should be considered in further studies.

LBP.01

Extracellular vesicles isolated from the liver accelerate recovery of carbon tetrachloride-induced hepatic necrosis

Lee Changjin, Sae Rom Kim, Yong Song Gho, Gyeongyun Go, Hyun Taek Park and Nhung Thi Hong. Dinh

POSTECH

Introduction: Liver transplantation is still a major treatment for end-stages of liver diseases. Cells such as hepatocytes have shown promise effects on both acute and chronic liver damages. However, issues on survival and differentiation of isolated hepatocytes are remained unsolved. Cells secrete proteolipid-enclosed extracellular vesicles (EVs) which exert important biological roles in intercellular communication. However, EVs from *in vitro* cell culture do not fully recapitulate the function of EVs present *in vivo* due to environmental differences of cells. Therefore, we here examined the characteristics of *in vivo* EVs isolated from fresh liver tissue, where hepatocytes make up 70-85% of their mass, and their therapeutic efficacy on a mouse model of carbon tetrachloride (CCl_4)-induced liver damage.
Methods: EVs were extracted from fresh mouse liver tissues. Combinational method comprising differential centrifugation, ultracentrifugation, and buoyant density gradient ultracentrifugation was employed in isolating EVs from crude liver tissue extract. Nanoparticle tracking, dynamic light scattering, electron microscopic, and immunoblotting analyses are used to characterize the liver EVs. To examine effect of liver EVs on damaged liver, mice intraperitoneally received with CCl_4 were subsequently treated with or without the purified liver EVs and time course experiments were performed. Multiple analyses such as blood markers for liver damages, histology of damaged liver tissues, and immunohistochemistry for several molecules are followed.
Results: EVs isolated from fresh liver tissues exhibited typical physicochemical characteristics of EVs regarding sizes around 100 nm in diameter, spherical morphology, density of 1.14 g/ml, and enrichment of tetraspanins. Exogenous application of liver EVs to the mouse received with CCl_4 has shown that 1) rapid decrease of blood levels of liver damage makers, ALT, AST, and LDH that are elevated upon CCl_4 treatment, 2) early recovery of necrotic lesion in damaged liver, 3) suppression of apoptotic progression, and 4) spatial elevation of hepatocyte growth factor as compared to the animal not received with liver EVs.
Summary/Conclusion: Collectively, we suggest that the liver EVs have of great potentials as a new type of intervention especially for liver injuries.

LBP.02

Role of human corneal keratoinocyte-derived extracellular vesicles in corneal wound healing

Aleksandra Leszczynska[1], Mangesh Kulkarni[1], Kavita Patel[1], Talia Barkhordari[1], Nima Natanzi[2] and Mehrnoosh Saghizadeh Ghiam[1]

[1]Cedars-Sinai Medical Center, CA, USA; [2]Cedar-Sinai Medical Center, CA, USA

Introduction: The interaction between stromal keratinocytes and the epithelial cell is known to provide supportive mechanism to repair the injured epithelial cells. Traditionally, this interaction has been shown to be mediated by paracrine factors. We now know that extracellular vesicles (EVs) are bioactive molecules that play important role in cell communication and many physiological processes during wound healing and regeneration. We hypothesized that corneal keratinocyte-derived EVs (kerato-EVs) deliver the supportive miRNA to injured limbal epithelial cells (LECs) and that disease states such as diabetes affects their ability to deliver factors to target cells for tissue regeneration after injuries.
Methods: EVs were isolated from normal (N) and diabetic (DM) primary keratinocytes by ultracentrifugation or using Exoquick precipitation kit. Their size and number of the vesicles was confirmed by Nanosight. We also assessed the expression of EVs markers CD63 and CD81 on N and DM kerato-derived EVs by flow cytometry using magnetic beads. Proliferation was done by MTS assay and migration was checked by *in vitro* scratch assay.
Results: The number of EVs isolated from normal keratinocytes was an order of magnitude higher than from DM samples. We showed the expression of EVs markers CD63 and CD81 on N and DM keratinocyte-derived EVs by flow cytometry using magnetic beads. Transwell migration assay performed with Dil labeled keratinocytes showed that EVs can migrate from keratinocytes to epithelial cells. Thus, we observed an active transfer of EVs. Simultaneously, direct addition of labeled EVs was performed as controls. There was greater uptake of N Kerato-EVs than DM Kerato-EVs. Results of MTS assay showed that both N and DM keratinocyte derived EVs induced proliferation in human corneal epithelial cells (HCEC); to a greater extent by N vs. DM keratinocyte-derived EVs. We performed *in vitro* scratch assay on HCECs that were treated with N and DM keratinocyte-derived exosomes. The results demonstrated that the migration of HCECs increased at 24h in presence of EVs as compared to control group.
Summary/Conclusion: We have demonstrated that EVs derived from keratinocytes are taken up by corneal epithelial cells even without direct contact. Also, we have shown that Diabetes affects the production of EVs from corneal keratinocytes and also their ability to affect proliferation and migration of epithelial cells.
Funding: CSMC

Poster Session PT04 – EVs in Cancer Therapy and Drug Resistance
Chairs: Jun Chung and Mary Bebawy 5:15–6:30 p.m.

PT04.01

Withdrawn at author's request.

PT04.02

EVs in cisplatin resistance and transmitting resistance in calu1 non-small cell lung cancer cells

Ilgin Kisiogu[1], Gokce Lara Bodur[1] and Mustafa Kotmakçı[2]

[1]Ozel Ege High School, Bornova, Izmir, Turkey; [2]Department of Pharmaceutical Biotechnology, Ege University, Bornova, Izmir, Turkey

Introduction: In recent decades, extracellular vesicles (EVs) were shown to play important roles in a plethora of biological processes, including chemoresistance development and transmission between cancer cells. The aim of this study was to investigate whether EVs isolated from cisplatin-resistant Calu1 (CR-Calu1) cells transport the drug out of the cell cytoplasm, and to study the effect of isolated EVs on the parental Calu1 cells.
Methods: CD-Calu1 cells were previously developed by incubating of Calu1 cells in culture medium containing cisplatin at continuously increasing concentrations. CD-Calu1 cells were maintained in DMEM containing 100 μM cisplatin. 48 h prior to EV isolation, culture medium was replaced with fresh EV-free DMEM. EVs were isolated by sequential centrifugation followed by ultracentrifugation at 120,000*g*. Protein concentration of EVs was measured with Bradford protein assay. Particle size measurement of EV isolate was perfotmed by dynamic light scattering. Presence of cisplatin in EV isolates was analysed by X-ray photoelectron spectroscopy (XPS) analysis of Pt 4f. This method has a sensitivity of 0.1% atomic percentage. Transmission of drug resistance to sensitive cells was investigated by simultaneous administration of EVs and cisplatin to native Calu1 cells. Cell viability was investigated by XTT cell proliferation assay and trypan blue exclusion.
Results: DLS results revealed that isolated vesicles vere of exosome and microvesicle type, according to the peak values at 44 and 295 nm, respectively. XPS measurements revealed that EVs isolated from CR-Calu1 cells do not contain cisplatin, which was supported by the absence of Pt 4f doublet peak at 80–70 eV of XPS spectrum. Cisplatin at 20 μM dose reduced viability of Calu1 cells to approx. 40%, while coadministration of CR-Calu1 EVs and cisplatin reduced viability of native Calu1 cells to approx. 80%.
Conclusion: Cisplatin resistance in Calu1 cells does not seem to be accompanied by excretion with EVs. EVs from cisplatin resistant Calu1 cells increased viability of native Calu1 cells in the presence of cisplatin. Further investigatinon of molecules responsible for transmission of the resistance in cisplatin resistant Calu1 cells can provide better therapeutic strategies for lung cancer.

PT04.03

Paclitaxel-loaded milk exosomes overcome immunotoxicity following oral administration

Ashish Kumar Agrawal[1], Farrukh Aqil[2], Jeyaprakash Jeyabalan[1], Varun Kushwah[1], Wendy Spencer[3], Josh Beck[3], Beth Gachuki[4], Sarah Alhakeem[4], Karine Oben[4], Radha Munagala[2], Subbarao Bondada[4] and Ramesh C. Gupta[5]

[1]JG Brown Cancer Center, University of Louisville, Louisville, KY, USA; [2]Department of Medicine and JG Brown Cancer Center, University of Louisville, KY, USA; [3]3P Biotechnologies, Inc., Louisville, KY, USA; [4]Department of Microbiology, Immunology & Molecular Genetics and Markey Cancer Center, University of Kentucky, Lexington, KY, USA; [5]Department of Pharmacology and Toxicology and JG Brown Cancer Center, University of Louisville, Louisville, KY, USA

Introduction: Paclitaxel (PAC) has been recognised as a first-line treatment for various cancers. However, severe toxicities associated with the conventional *i.v.* therapy, and its carrier Cremophor EL, make it disadvantageous for many patients. Here we investigated exhaustively immunotoxicity of PAC-loaded exosomes (ExoPAC) following oral administration, as well as potential mechanism of drug loading.
Methods: ExoPAC was prepared by mixing the PAC solution (in ethanol: acetonitrile, 1:1) with milk exosomes (Exo), and the particle size was measured by zetasizer, and the mechanism of drug loading studied by fluorescence spectroscopy. *In vitro* release of PAC from ExoPAC was determined in simulated-gastrointestinal fluids and PBS. To determine potential toxicity, wild-type female C57BL/6 mice were treated with PBS, Exo (80 mg/kg), and ExoPAC (12 mg/kg) by oral gavage, five times a week, and PAC *i.v.* (12 mg/kg) once a week. After three weeks, animals were euthanised and blood and select tissues were collected to measure immunotoxicity.
Results: High PAC loading was observed due to hydrophobic interactions between PAC and Exo proteins as principal mechanism of drug loading based on significant quenching of fluorescence of the native Exo, particle size of ExoPAC was somewhat increased compared with Exo (75 vs. 108 nm). ExoPAC showed excellent physicochemical stability under simulated conditions. The PAC was released time-dependently – ≈20% in case of FeSSGF after 2 h, ≈40% in FeSSIF after 4 h and >90% in PBS, after 48 h, suggestive of a minimal effect of pH and different enzymes present in the FeSSGF and FeSSIF. A significant reduction in immune toxicity was observed with orally administered ExoPAC vs. PAC *i.v.* based onimmune cell quantification by single cell suspension of spleen cells and flow cytometry analysis of bone marrow stem and progenitor cells.
Conclusion: Rigorous data on multiple immunological parameters rule out the immunological adverse effects due to foreign biological material and cross-species reaction; in fact, PAC administered orally as an exosomal formulation seems to overcome adverse immunological effects associated with PAC *i.v.* treatment.

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PT04.04

Transference of resistance phenotype mediated by extracellular vesicles in gastric cancers

Edson Kuatelela Cassinela, Gabriela Pintar de Oliveira, Antuani Baptistella, Fernanda Giudice, Michele Christine Landemberger; Fabio Marchi and Vilma Regina Martins

A.C. Camargo Cancer Center, Sao Paulo, Brazil

Introduction: Gastric adenocarcinoma (GAd) is one of the most common cause of cancer death worldwide and one of the tumours with higher mortality rates in Brazil. The mechanisms of GAd pathogenesis are largely unknown what causes limitations in the personalised treatment and neoadjuvant therapy has been largely applied in these tumours because it can improve tumour resectability and survival of patients. However, tumours develop resistance to chemotherapy, which is the major reason for the failure of treatment. Indeed, the understanding of the mechanisms associated to chemotherapy resistant is of great relevance.
Methods: A GAd cells line (AGS) was used to generate a cell line resistant to 5-fluorouracil (rAGS_FU). Extracellular vesicles (EVs) secreted from AGS and rAGS_FU cell lines were isolated by ultracentrifugation, quantified and evaluated regarding their aggressiveness through invasion assays. Proteomics and Next generation sequencing analysis of EVs and secreting cells were also performed
Results: rAGS_FU cells secrete more EVs and presented increased invasion rates than AGS cells. Extracellular vesicles (EVs) derived from rAGS_FU cells were able to promote resistance to chemotherapy and to induce an increase in invasion in AGS cells. Thus, cells resistant to chemotherapy have a more aggressive phenotype and are able to transfer this acquired characteristics to the non-resistant ones using EVs. Proteomics analysis revealed that proteins involved in resistance to therapy such as FSCN1, are overexpressed or exclusively expressed in rAGS_FU EVs when compared to

AGS cells. Next generation sequencing analysis of the entire transcriptome (long and short RNAs) from EVs and secreting cells revealed transcripts differentially expressed between AGS and rAGS_FU cell lines such as hsa-miR-181a-5p and hsa-miR-372-3p which may be directly or indirectly, involved in the resistant phenotype.
Conclusion: A deep investigation of these data is needed to understand and create new opportunities for the discovery of new biomarkers of response to chemotherapy in gastric cancers and contribute to the better understanding of the biological role of molecules shuttled by EVs.

PT04.05

Exosomal delivery of small molecules for the management of ovarian cancer

Farrukh Aqil[1], Jeyaprakash Jeyabalan[2], Radha Munagala[1], Ashish Kumar Agrawal[2], Lynne Parker[3] and Ramesh C. Gupta[4]

[1]Department of Medicine and JG Brown Cancer Center, University of Louisville, Louisville, KY, USA; [2]JG Brown Cancer Center, University of Louisville, Louisville; [3]Norton Healthcare Pavilion, Louisville, KY, USA; [4]Department of Pharmacology and Toxicology and JG Brown Cancer Center, University of Louisville, Louisville, KY, USA

Introduction: Ovarian cancer is the fifth deadliest cancer among US women. Resistance to chemotherapy, lack of oral bioavailability and off-site toxicity of chemo drugs present major obstacles in the treatment of patients with ovarian cancer. We hypothesised that drug molecules administered via exosomes will increase their oral bioavailability, and folic acid (FA)-functionalised exosomes will further enhance therapeutic response and reduce off-target toxicities.
Methods: Exosomes (Exo) were isolated from bovine milk and their size was measured by zetasizer. Small drug molecules (withaferin A (WFA), anthocyanidins (Anthos) and paclitaxel (PAC)) were loaded onto the Exo. Antiproliferative activity of Exo formulations was determined against ovarian cancer drug-sensitive (A2780) and drug-resistant (OVCA432) cells. Anti-tumour activity was determined against A2780 tumour xenografts in nude mice delivering the Exo formulations by oral gavage, except PAC which was given *i.p*. Tumour targeting was achieved by co-loading of the tumour-targeting ligand, FA.
Results: The isolated Exo showed the size of 93 ± 8 nm. Test agents (WFA, Anthos and PAC) could be loaded onto Exo with 8–20% drug load. ExoWFA and ExoAnthos showed significantly higher (2–10 fold) antiproliferative activity versus the free drugs, antiproliferative activity of ExoPAC was only slightly higher than free PAC. Anthos and WFA both demonstrated modest but insignificant anti-tumour activity. However, the tumour growth inhibition was significantly higher with the ExoAnthos (65%) and ExoWFA (60%), which was further enhanced when these formulations were functionalised by FA. Similarly, ExoPAC administered orally showed the same therapeutic efficacy as free PAC given *i.p*. However, significantly higher antitumor activity was achieved when the ExoPAC was FA-functionalised. A modest, but insignificant tumour inhibition was also observed with the Exo alone. Our data showed significantly enhanced antitumor activity by ExoPAC formulation when combined with ExoAnthos or ExoWFA.
Conclusion: Our data indicate that the milk-derived exosomes serve as excellent nano-carriers for small drug molecules to enhance oral bioavailability against ovarian cancer.

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PT04.06

Evaluation of drug resistance transfer via extracellular vesicles in human ovarian cancer cells

Jennifer F. Power and Susan P.C. Cole

Department of Pathology & Molecular Medicine, Queen's University, Ontario, Canada

Ovarian cancer (OCa) is the fifth most common cancer and has the highest mortality rate of all gynaecologic malignancies. Symptoms of early stage OCa are rarely detectable resulting in late stage diagnoses and poor prognoses. First-line chemotherapy of OCa includes paclitaxel (PXL) and carboplatin. Unfortunately, patients almost always relapse with drug-resistant disease, resulting in 5-year survival rates < 45%. Extracellular vesicles (EVs) can facilitate cell–cell communication, and have been implicated in promoting cancer growth and metastasis, as well as drug resistance. Resistance can be caused by many mechanisms including elevated levels of the ATP-binding cassette (ABC) drug efflux transporters P-glycoprotein/ABCB1 (P-gp), MRP1/ABCC1, and/or ABCG2/BCRP. Our aim is to determine whether resistance via an ABC transporter may be transferred by EVs derived from OCa cells. Paired sensitive and resistant human OCa cell lines (parental A2780-9S and resistant A2780-AD645) were cultured under standard conditions. The relative resistance of A2780-AD645 cells was determined by sulforhodamine B cytotoxicity assays after 48 h drug exposure. Cells were grown in EV-free media for 24 h prior to collection of conditioned media and EVs isolated by differential centrifugation. Fractions collected at 20,000*g* (20 K) and 100,000*g* (100 K) were solubilised and immunoblotted for P-gp and established EV tetraspanin markers CD63 and CD81. Cytotoxicity assays confirmed that A2780-AD645 cells were 17-fold and >50-fold resistant to doxorubicin and PXL, respectively, and elevated P-gp levels were detected in whole cell and membrane enriched extracts by immunoblot, as expected. CD63 and CD81 were readily detected and highly enriched in the 100 K fraction but only CD63 was detected in the 20 K fraction, as well as in whole cell and membrane enriched extracts. Our results indicate the feasibility of using OCa cell lines to explore how EVs might mediate drug resistance. Ongoing studies include optimising P-gp detection in EVs, co-culture assays to determine if EVs from resistant OCa cells can reduce the sensitivity of parental cells, and identification of the messenger(s) in the EVs (i.e. protein, nucleic acid) responsible.

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PT04.07

Extracellular vesicles confer a complex multidrug resistance and survival profile in cancer through the transfer of drug efflux capacity, drug sequestration, metastasis, altered tissue biomechanics and immune evasion

Deep Pokarel, Jamie Lu, Jack Taylor, Ariane Roseblade, Sabna Rajeev Krishnan and Mary Bebawy

The Graduate School of Health, The University of Technology Sydney, Sydney, Australia

Multidrug resistance (MDR) contributes to treatment failure in over 90% of patients with metastatic cancer. MDR is a unique type of resistance in which cancer cells become cross-resistant to a wide range of drugs used in combination chemotherapy. Synonymous with this phenotype is the overexpression of plasma membrane drug transporters which efflux drugs out from cancer cells. These transporters limit the intracellular accumulation of chemotherapeutics by virtue of ATP dependent drug efflux, rendering cancer cells unresponsive to treatment.
We discovered that extracellular vesicles, specifically, microparticles (MPs), provide a novel pathway(s) for the dissemination and acquisition of cancer MDR. This occurs through the intercellular transfer of functional resistance proteins and nucleic acids and through a capacity for active and passive drug sequestration by MPs. We have also shown that MPs derived from MDR cells readily confer the donor cell traits within recipient cancer cell populations, including MDR, enhanced metastatic capacity and altered tissue biomechanical properties. Our most recent studies demonstrate the presence of a distinct and parallel MP meditated pathway supporting the survival of MDR cancer cells through immune evasion.
These findings provide the necessary insight and basis for the design of novel therapeutic strategies, targeted at the prevention and circumvention of MDR clinically. From a clinical perspective, these results have recently led us to establish MPs as valuable systemic biomarkers for assessing treatment responsiveness, risk of relapse and the evolution of disease in individual myeloma patients.

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PT04.08

Direct effects of anti-angiogenic therapies on glioblastoma cells interactions with astrocytes via extracellular vesicles

Thomas Simon, Sotiria Pinioti, Franz Wendler and Georgios Giamas

University of Sussex, Brighton, United Kingdom

Introduction: Glioblastoma (GBM) is the most aggressive type of primary brain tumours in humans. Hence, anti-angiogenic therapies (AAT) have been developed to target the tumour blood supply in order to reduce its invasiveness. However, mechanisms of AAT-resistance have been observed. Among them, an effect of AAT directly on GBM cells through the blocking of autocrine signalling, such as VEGF signalling, has been speculated but still remains unknown. We believe that such direct effect could affect the tumour cells communication with their stromal counterparts, including astrocytes, through secreted extracellular vesicles (EVs). Such alterations in the GBM cells relationships with their microenvironment in response to AAT could be involved in therapeutic resistance.
Methods: Human astrocytes and GBM cell lines were treated with three different AAT. Amount of EVs produced by astrocytes and GBM cells following treatments with AAT were quantified. Mass spectrometry and western blotting were used to characterise EVs protein content. In particular, effects of AAT and EVs from AAT-treated GBM cells on the phenotype of astrocytes (paracrine) and GBM cells (autocrine) were being examined.
Results: Direct inhibitory effects of two out of three AAT have been observed on astrocytes and GBM cells viability. In addition, alterations in the amount of EVs produced by astrocytes and GBM cells have been noticed in response to AAT. Furthermore, it appears that EVs derived from AAT-treated cells can affect astrocytes and GBM cells viability. Finally, in EVs from AAT-treated cells, proteomic analyses identified protein hits that could be involved in GBM aggressiveness.
Conclusion: According to the type of drug, GBM cells and astrocytes are differently affected by AAT. In addition, regarding the effects of EVs from AAT treated-GBM cells on other GBM cells and astrocytes phenotype, we suggest that EVs-driven communication between GBM cells and astrocytes could be affected following AAT treatment. Further proteomic and genomic analyses are needed to decipher the molecular mechanisms underlying such effects. Consequently, this study can bring new insights about a potential "direct" effect of AAT on GBM cells during therapeutic resistance.

PT04.09

Analysis of the fate of chemotherapeutic drugs expelled by pancreatic cancer cells into microvesicles

Vandhana Muralidharan-Chari and Shaker Mousa

Albany College of Pharmacy and Health Sciences, NY, USA

Introduction: High mortality in pancreatic cancer patients is partly due to resistance to chemotherapy. We identified that pancreatic cancer cells utilise microvesicles (MVs) to expel and remove chemotherapeutic drugs. Using human pancreatic cancer cells that exhibit varied sensitivity to gemcitabine (GEM), we showed that GEM exposure triggers the cancer cells to release MVs in an amount that correlates with that cell line's sensitivity to GEM. The inhibition of MV release sensitised the GEM-resistant cancer cells to GEM treatment, both *in vitro* and *in vivo*. Mechanistically, MVs remove drugs that are internalised into the cells and that are in the microenvironment. We also explained the differences between the GEM-resistant and GEM-sensitive pancreatic cancer cell lines tested based on the variable content of GEM-transporter proteins, which control the ability of MVs either to trap GEM or to allow GEM to flow back to the microenvironment. In this study, we describe the fate of GEM that has been expelled by the cells into the MVs.
Methods: Human pancreatic cancer cells were treated with GEM, and MVs were isolated at various time points. The presence of GEM-metabolising enzymes within the isolated MVs was analysed with western blotting techniques. MV-lysates were further analysed for the activity of the metabolising enzymes, and their by-products were analysed with HPLC-MS/MS analysis.
Results and Summary: We show data for the first time of the presence of metabolising enzymes and their by-products within MVs released by pancreatic cancer cells upon exposure to GEM. Data are compared between GEM-resistant pancreatic cancer cells and GEM-sensitive pancreatic cancer cells, and the significance of the results will be discussed in the context of biological relevance of the presence of GEM within the released MVs, given that MVs can fuse with various cell types in the body.

Poster Session PT05 – Novel Developments in EV Characterisation
Chairs: Matias Ostrowski and Sten Libregts 5:15–6:30 p.m.

PT05.01

Raman tweezers microspectroscopy of single extracellular vesicles: towards measuring the relative content of proteins, lipids, and nucleic acids

Sergei G. Kruglik[1], Irène Tatischeff[2], Pierre-Yves Turpin[1], Jean-Michel Guigner[1], Félix Royo[3] and Juan M. Falcón-Pérez[3]

[1]University Pierre & Marie Curie Paris 6, France; [2]Consulting company REVINTERCELL; [3]CIC bioGUNE

Introduction: Extracellular vesicles (EVs) contain a wealth of information on health and disease, possessing a great potential in theranostics of cancer (1). In addition to «omics» techniques sensitive to individual biomolecules, Raman tweezers microspectroscopy (RTM) is arising as a label-free analytic tool, providing information on global biomolecular composition of EVs, especially promising in combination with cryogenic transmission electron microscopy (Cryo-TEM) (2). Our study focuses on potentialities of RTM for rapid characterisation of single (or very few) EVs through their specific biomolecular content.
Methods: In RTM experiment, bioparticles were optically trapped by a strong 785-nm laser beam and their Raman spectra were analysed for the presence of major constituent biomolecules. We studied EVs released by *Dictyostelium discoideum* cells, mouse cell line MLP29, and primary rat hepatocytes, as well as extracted from human urine (exosomes). Size distribution of all studied bioparticles was characterised by Cryo-TEM.
Results: The RTM technique was optimised for EVs in 50–200 nm size range, and high-quality Raman spectra of single (or a few, depending on particles size) EVs were obtained for all samples. Contributions from proteins, lipids and nucleic acids were analysed, and their relative content was estimated. The lower concentration limit for Raman detection of biomolecules in water is in the order of a few mM, however, due to the effect of optical trapping in the focus of a laser beam, RTM can effectively detect high local concentrations of biomolecules in trapped EVs.
Conclusion: RTM is a promising tool for characterisation of individual EVs on relative biomolecular content. One immediate application, among many others, is discrimination between EVs, lipid bodies, protein aggregates, and possibly viruses, in hardly accessible 100 nm size range.

References

1. Tatischeff, *Cancer Res. Front.* 2015; 1: 208.
2. Tatischeff et al., *JEV* 2012; 1: 19179.

PT05.02

Magnetic nanoparticle-enhanced surface plasmon resonance biosensor for extracellular vesicle analysis

Agnes T. Reiner[1], Ruenn Chai Lai[2], Sai Kiang Lim[2] and Jakub Dostalek[1]

[1]BioSensor Technologies, AIT-Austrian Institute of Technology GmbH, Seibersdorf, Austria; [2]A*STAR

Even though extracellular vesicles (EVs) are emerging as new tools in clinical applications for disease diagnosis, monitoring and treatment, reliable detection methods are still lacking. In this work we propose a biosensor with wavelength interrogation of grating-coupled surface plasmon resonance (SPR) for the analysis of EVs. In order to overcome diffusion-limited binding kinetics and allow for detection of trace amounts of vesicles present in complex samples, magnetic nanoparticles are employed for collecting the target analyte on the sensor surface. The grating-coupled SPR is demonstrated as an efficient platform, that allows pulling of the target analyte to the sensor surface by usage of a magnetic field gradient applied through the sensor chip. By this means, the sensor response is greatly enhanced by the more efficient yield in collecting and affinity binding of the target analyte on the sensor surface and by the magnetic nanoparticle-enhanced change in the surface mass density associated to the analyte capture. The capability of this sensor to detect EVs is demonstrated by the analysis of different EV populations derived from mesenchymal stem cells, which carry different lipid and protein moieties.

PT05.03

Probing nanosized extracellular vesicle (EV) populations by surface-enhanced Raman spectroscopy (SERS)

Lucia Paolini[1], Nicolò Bontempi[2], Annalisa Radeghieri[1], Anna Castelli[1], Andrea Zendrini[1], Sara Busatto[1], Eugenio Monti[1], Ivano Alessandri[2] and Paolo Bergese[3]

[1]Department of Molecular and Translational Medicine, University of Brescia, KY, USA; [2]Department of Mechanical and Industrial Engineering, Chemistry for Technologies Laboratory, University of Brescia and INSTM UdR Brescia, KY, USA; [3]Department of Molecular and Translational Medicine and INSTM UdR Brescia, University of Brescia, KY, USA

Introduction: Surface-enhanced Raman spectrosctopy (SERS) is a powerful resource to provide information about the biochemical content of extracellular vesicles (EVs) in a fast and reproducible way. We explored the ability of plasmonic and non-plasmonic SERS to probe nanosized EV populations separated from human serum of patients affected by multiple myeloma (MM) or Parkinson's disease (PD) and from healthy (H) donors. Typically, metal nanoparticles (NPs) with a plasmonic resonance (e.g. Au) are utilised to enhance the Raman response (plasmonic SERS). However, excited plasmonic NPs generate local heating and energy release, thereby inducing instability and low reproducibility, especially with organic or biological analytes. For this reason we also considered to probe EVs with innovative T-rex beads made of SiO_2/TiO_2 core/shell colloids that enhance the Raman fingerprint of the analyte by non-plasmonic SERS, thus expected to show a lower ability impact on the stability of the adsorbed EVs.
Methods: EVs from serum of H patients and those with MM or PD were purified using sequential centrifugation steps and discontinuous sucrose gradients. Samples were biochemically characterised by western blot analysis. Positive fractions to typical exosomal markers were pooled and further characterised for biophysical characteristics by atomic force microscopy (AFM), colloidal nanoplasmonic assays and an agarose gel. EVs were then targeted with 15 nm Au NPs and analysed by conventional SERS. In alternative EVs were coupled with T-rex beads for non-plasmonic SERS.
Results: The colloidal nanoplasmonic assay allowed us to assess purity and determine the molar concentration of the EV formulations, AFM imaging confirmed the formulation to be composed of nanosized EV populations (50–100 nm). Both plasmonic and non-plasmonic SERS experiments gave promising results in terms of the possibility to use SERS profiling to identify each of the H, MM and PD EV populations. Our contribution will focus on presenting and discussing the last updates of these results (further experiments are ongoing).
The institutional review board of *Azienda Ospedaliera Spedali Civili* of Brescia approved the study in adherence with the Declaration of Helsinki. This project was financed by the BIOMANE grant from the University of Brescia 2015.

PT05.04

Multiplexing characterisation of neuronal exosomes from human plasma by surface plasmon resonance imaging

Silvia Picciolini[1], Alice Gualerzi[2], Carlo Morasso[2], Renzo Vanna[2], Marzia Bedoni[3], Massimo Masserini[4] and Furio Gramatica[2]

[1]Laboratory of Nanomedicine and Clinical Biophotonics LABION, Fondazione Don Gnocchi – University of Milano-Biocca, Milano, Italy;

[2]Laboratory of Nanomedicine and Clinical Biophotonics LABION, Fondazione Don Gnocchi; [3]Laboratory of Nanomedicine and Clinical Biophotonics LABION, Fondazione Don Carlo Gnocchi ONLUS; [4]University of Milano-Biocca, Milano, Italy

Introduction: Exosomes have emerged as a new class of biomarkers of neurological disorders showing an involvement in neurodegenerative processes. The big interest in this field is supported by the fact that exosomes are able to cross the blood brain barrier and can thus offer the unique possibility to study the biochemical processes inside the central nervous system from a biofluid easy to access as human blood. Inspired by recent progresses in plasmonic biosensors that demonstrated their ability to detect exosomes from biological samples, we have designed a biosensor based on surface plasmon resonance imaging (SPRi) for the isolation of exosomes of neuronal origin and to study their membrane surface and interactions with specific biomolecules.
Methods: The SPRi microarray was optimised for the detection of different subpopulations of exosomes extracted by size-exclusion chromatography from plasma of healthy volunteers. Bare gold SPRi chips were coated with a self assembled monolayer and further activated by EDC/NHS chemistry, in order to be functionalised with different antibodies, deposited by automated microspotting. After exosomes injection on the SPRi chip, we evaluated the interaction between their membrane molecules and specific antibodies.
Results: The surface chemistry was optimised for the immobilisation of antibodies and we tested simultaneously different antibodies such as CD9 and CD63 that are generic exosomes markers, and CD171/L1 as neuronal marker. Once the exosomes were adsorbed on the chip, the injection of other antibodies was followed by a signal in correspondence of specific exosomes subpopulations, demonstrating the possibility to characterise exosome membranes with a sandwich approach.
Conclusion: These results suggest that the use of SPRi can help to simultaneously discriminate and immobilise different exosomes subpopulations and to evaluate the interaction with biomolecules, with a perspective of investigating biological role of these biomarkers.

PT05.05

Cryogenic-temperature electron microscopy imaging of extracellular vesicles shedding

Naama Koifman[1], Idan Biran[1], Anat Aharon[2], Benjamin Brenner[3] and Yeshayahu Talmon[1]

[1]Department of Chemical Engineering and the Russell Berrie Nanotechnology Institute (RBNI), Technion – Israel Institute of Technology, Haifa, Israel; [2]Department of Haematology, Rambam Health Care Campus; [3]Department of Haematology and Bone Marrow Transplantation, Rambam Health Care Campus

Please see OPT03.01

PT05.06

Membrane vesicles – examination of biophysical properties with atomic force microscopy

Melissa C. Piontek and Wouter H. Roos

Zernike Institute for Advanced Materials, University of Groningen, Groningen, The Netherlands

Extracellular vesicles (EVs) are not only intensively studied to increase our fundamental knowledge on their functioning, but also for diagnosis, therapeutics and drug delivery purposes. To improve the current and potential applications of EVs, a fundamental understanding of their stability, structure, and function is crucial. Such studies can be conducted at the single particle level to gain biological and physical information about the vesicles and the particle to particle variability. A suitable technique to investigate EVs under near-to physiological conditions is atomic force microscopy (AFM). Operated in liquid, it provides images of the EVs while mechanical properties of the particles can be obtained as well. Here we present our approach and the latest results in studying the structure and mechanics of these particles.

Funding: This work is supported by NWO through a Vidi grant and by STW through the Perspectief grant Cancer-ID. (Both to Wouter H. Roos).

PT05.07

Detection and characterisation of exosomes in TEM images using ExosomeAnalyzer: a novel software tool

Anna Kotrbová[1], Karel Štěpka[2], Martin Maška[2], Jakub Jozef Pálenik[2], Ladislav Ilkovics[3], Dobromila Klemová[3], Aleš Hampl[3], Vítězslav Bryja[1], Vendula Pospíchalová[1] and Pavel Matula[2]

[1]Department of Experimental Biology, Faculty of Science, Masaryk University, Czech Republic; [2]Centre for Biomedical Image Analysis, Faculty of Informatics, Masaryk University, Czech Republic; [3]Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Czech Republic

Introduction: Exosomes (exs) are nano-sized extracellular vesicles that function as conveyers of information between cells. Their content reflects the cell of their origin and its condition. Different cargo of vesicles underlines their function and may have effect on morphological characteristics (shape, size) of exs. So far, there has been a lack of studies correlating morphological characteristics of vesicles with their content and possible function. This is caused partially by the fact, that analysis of individual exosomes in electron microscopy images is time-consuming if performed manually. Therefore we present here a software for computer-assisted evaluation of exosomes in TEM images.
Methods: Exosomes were isolated using differential centrifugation followed by a purification step in sucrose/D_2O cushion. Morphology of exs was observed using negative contrasting followed by visualisation using TEM. Morphological characteristics were analysed by ExosomeAnalyzer software based on their shape and edge contrast criteria. The exosome segmentation was carried out using morphological seeded watershed on gradient magnitude image, with the seeds established by applying a series of hysteresis thresholdings, followed by morphological filtering and cluster splitting.
Results: We developed a software tool capable of analysing morphological features of exs (size and roundness) in often not so clear TEM images. Even images with exs both lighter and darker than the background, or containing artefacts or precipitated stain, can be successfully processed. If the fully-automatic processing fails to produce correct results, the program allows the user to adjust the detection seeds as well as exosome boundaries manually.
Conclusion: Our software is an easy to use tool that might be of high interest to the ISEV community. It is publicly available at: http://cbia.fi.muni.cz/exosome-analyzer.

Funding: Grant Agency of Masaryk University (MUNI/M/1050/2013).

PT05.08

Immunogold labelling of extracellular vesicles and liposomes in the liquid phase

Naama Koifman[1], Maayan Nir-Shapira[1], Idan Biran[1], Anat Aharon[2], Benjamin Brenner[3] and Yeshayahu Talmon[1]

[1]Department of Chemical Engineering and the Russell Berrie Nanotechnology Institute (RBNI), Technion – Israel Institute of Technology, Haifa, Israel; [2]Department of Haematology, Rambam Health Care Campus; [3]Department of Haematology and Bone Marrow Transplantation, Rambam Health Care Campus

Introduction: Extracellular vesicles (EVs) have sizes ranging from tens of nanometres to >1 μm and carry a variety of membrane antigens emanating from their original cells. The detection of such compositional markers is of great importance both diagnostically and mechanistically. Immunogold labelling in transmission electron microscopy (TEM) utilises the high electron density of gold nanoparticles conjugated to antibodies. Cryogenic temperature-TEM (cryo-TEM) enables a single-vesicle examination, probing specific molecules on EVs, while covering the whole range of EV diameters, and preserving their nanostructure.
Methods: 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-l-serine (DOPS) liposomes were

prepared by extrusion, and used as model systems for the labelling optimisation. Labelling included a two-step process using biotinylated annexin-V and gold-conjugated streptavidin. We labelled different cell lines for annexin, and compared both the labelling levels and the morphology of the labelled vesicles. EVs isolated from platelets-rich plasma were used as a positive control for the presence of annexin-V. Antigens on cells of origin and on the EVs fraction were detected using flow cytometry.
Results: We selectively labelled DOPS liposomes versus DOPC liposomes. DOPS liposomes were shown to form aggregates in the presence of binding buffer due to the high electrostatic forces formed by the presence of Ca^{2+} ions on the surface of the DOPS-rich liposomes. Various annexin-V labelling levels were observed on EVs isolated from different cells lines. Preliminary results from THP1-isolated EVs show that only a fraction of the EVs present extensive immunogold-labelling for annexin-V. We have also attempted to label CD-14 on EVs isolated from monocytes and EGFR on EVs from MDA468.
Conclusion: The results present promising beginning for the development of a simple labelling technique, focusing on the pivotal issue of the lipid content of EVs. This entire methodology is carried out in the liquid phase, avoiding drying artefacts. Immunogold labelling in cryo-TEM of extracellular vesicles grants highly important information as to the morphology of the vesicles, paving the way for a high-resolution diagnostic method at a single-vesicle level.

PT05.09

Monitoring the progression of cell death and detailed characterisation of apoptotic bodies by flow cytometry

Lanzhou Jiang, Rochelle Tixeira, Stephanie Paone, Sarah Caruso, Georgia Atkin-Smith, Amy Baxter, Mark Hulett and Ivan Poon

La Trobe Institute for Molecular Science, Melbourne, Australia

More than 200 billion cells undergo apoptosis every day in human bodies. It is an integral part of the maintenance of tissue homeostasis. It is also related to many diseases such as systemic lupus erythematosus. During apoptosis, cells will break apart and form numerous membrane-limited vesicles known as apoptotic bodies. Recently, we have developed a new protocol based on flow cytometry which can accurately differentiate apoptotic bodies from other particles in a mixed sample. This protocol uses a combination of Annexin A5 and TO-PRO-3 (a commercially available nucleic acid-binding dye that stains early apoptotic and necrotic cells differentially), and a logical seven-stage analytical approach to distinguish six types of particles in a sample, including apoptotic bodies and cells at three different stages of cell death. The method can be used to study the characteristics of apoptotic bodies in details, especially how cellular contents are distributed into the apoptotic bodies and how to trace biomarkers that indicate the origins of apoptotic bodies. For example, to study organelle distribution, we can use a combination of intracellular organelle staining (such as Hoechst, Mitotracker green, Lysotracker red etc.) and correlated stains such as TO-PRO-3 and Annexin A5-V450/Annexin A5-FITC etc. The established methodologies can enable us to better characterise the apoptotic cell disassembly process, which is a key downstream process of cell death.

PT05.10

Novel triggering threshold strategy for discovery of rare microvesicle phenotypes on flow cytometers dedicated to small particle analysis

Mathilde Sanden, Jaco Botha, Morten Hjuler Nielsen and Aase Handberg

Department of Clinical Biochemistry, Aalborg University Hospital, Aalborg, Denmark

Introduction: Detection and characterisation of microvesicles (MVs) have clinical relevance as they can function as potential biomarkers for diseases. Recent advances have led to the development of flow cytometers dedicated to the detection and characterisation of small particles. However, current protocols are insufficient as they are developed and optimised for conventional flow cytometers. Aim: To compare the purity and quantity of phosphatidylserine-exposing (PS+) MVs between different triggering threshold strategies to determine optimal settings for discovery and quantification of rare MV phenotypes.
Methods: Size-calibrated green fluorescent silica beads were used to determine the MV-regions on the Apogee A60-Micro PLUS flow cytometer. Plasma from one healthy donor was labelled with Lactadherin-FITC, CD41-APC and CD36-PE. Three different threshold strategies were examined: threshold on light scatter; fluorescence; light scatter and fluorescence combined.
Results: The number of PS+, CD36+/CD41+, CD41+ or CD36+ MVs did not differ between the three threshold strategies. Large differences were observed in total number of events and file sizes between light scatter (3.65×10^5, 50.1 Mbyte), fluorescence (0.40×10^5, 5.59 Mbyte) and combined (0.14×10^5, 1.87 Mbyte) strategies. Serial dilutions indicated linearity for all three strategies suggesting that swarm detection is unlikely ($R^2 = 0.957–0.999$).
Conclusion: The sensitivity of dedicated flow cytometry is sufficient to detect comparable numbers of PS+ MVs and different phenotypes regardless of the thresholding strategy. However, thresholds on both light scatter and fluorescence is the most optimal strategy allowing data acquisition over longer periods of time, thereby increasing the purity and quantity by collecting more specific events with a minimised file size. These initiatives render dedicated flow cytometry more suitable to discover rare MV phenotypes and thereby more specific and sensitive biomarkers.

PT05.11

Non-linearities in nanoscale flow cytometry of extracellular vesicles and standards

Janice Gomes[1], Fabrice Lucien[2], Christopher McIntyre[3] and Hon Sing Leong[2]

[1]University of Western Ontario, Ontario, Canada; [2]Lawson Health Research Institute, Ontario, Canada; [3]London Health Sciences Centre, London, United Kingdom

Introduction: Extracellular vesicles (EVs) have gained tremendous attention within the scientific community in recent years as these submicron particles have shown to be involved in many pathological conditions and diseases. Isolation and analysis of EVs from various bodily fluids represent an important challenge because there are currently few standardised methods that have been established. In contrast to conventional flow cytometry, nanoscale flow cytometry allows for analysis of particles that are between 100–1000 nm, while still utilising similar properties such as forward and side angle light scatter and more sensitive photomultiplier tubes. Even though nanoscale flow cytometry is an exquisite tool for EV analysis, improvements are still necessary to limit "swarm effect" and the false quantification of "true events" in samples. Our study aims to identify improvements to nanoscale flow cytometry and reduce inaccurate linearity associated with extracellular vesicles and standards.
Methods: We utilised the A50-Micro nanoscale flow cytometer (Apogee FlowSystems Inc.) to identify and measure 100–1000 nm sized extracellular vesicles and standards. We used patient plasma, conditioned media, latex beads, and silica beads at successive dilutions to determine the events based on forward and side angle light scatter, as well as quantification established by fluorescent markers
Results: We found that solely using forward and side angle light scatter was limiting and produced non-linear results following serial dilutions of patient plasma and conditioned media, and this further resulted in false EV quantification. Additionally, we found that while the threshold is a useful parameter to eliminate noise and undesired events without eliminating true events, adjusting the threshold of the fluorescent channels was more effective than merely the threshold of forward and side angle light scatter parameters.
Conclusion: While nanoscale flow cytometry is a major advancement in the identification of EVs at a submicron level, our results suggest that optimising functions such as threshold, and utilising fluorescent labelling for enumeration of EVs will result in a more accurate estimation of observed events.

PT05.12

Using flow cytometry and imaging flow cytometry to resolve the heterogeneity of extracellular vesicles including exosomes

André Görgens[1,2], Michel Bremer[2], Giulia Corso[1], Ulrika Felldin[1], Rita Ferrer-Tur[2], Dhanu Gupta[1], Helmut Hanenberg[3], Joel Z. Nordin[1], Helena

Sork[1], Svenja Meiler[4], Stefan Wild[4], Bernd Giebel[1,2] and Samir EL-Andaloussi[1,5]

[1]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden; [2]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; [3]Department of Pediatrics III, University Children's Hospital Essen, University of Duisburg-Essen, Essen, Germany; [4]Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; [5]Department of Physiology, Anatomy and Genetics, University of Oxford, United Kingdom

Extracellular vesicles (EVs) can be harvested from cell culture supernatants and from all body fluids. They can be roughly classified based on their size and origin as exosomes (70–150 nm) which are released when multivesicular bodies fuse with the plasma membrane, and microvesicles (100 nm to 1 μm) which are formed by the outward budding of the plasma membrane. In addition to these different EV subtypes, it is nowadays commonly accepted in the field that there is a much higher degree of EV heterogeneity within these two subgroups. The content, the protein composition and the surface signature of EVs vary and are likely to be dependent on the cell type source, the cell's activation status and multiple other parameters. Until today, no specific markers to discriminate even exosomes from microvesicles have been identified, and only few EV surface markers have been related to specific cell sources. In general, the question of heterogeneity in EV samples is rarely addressed at the experimental level, mainly due to the lack of qualified methods to analyse multiple parameters of single EVs. However, the identification of specific vesicular surface markers will be of high relevance to further understand the molecular content and related functions of subsets of EVs.
In the last few years, we and others developed different multi-parameter methods for flow-cytometric analysis of EVs, including bead-capturing methods. Of note, we recently optimised an imaging flow cytometry-based method and demonstrated its use to analyse multiple parameters on single exosomes in heterogeneous samples. Now, we have started to apply those flow cytometric approaches to analyse EVs derived from various sources, including cell lines of mesenchymal, epithelial, endothelial and hematopoietic origin. First, we are applying a multiplex bead-based method to screen for new EV surface markers. In a second step, we will validate newly identified markers at the single vesicle level by using imaging flow cytometry. Here, we present preliminary results obtained, and with this study we expect to further unravel heterogeneity of EVs and identify new and cell source specific EV surface signatures.

PT05.13

The use of a violet laser (405 nm) for scatter detection of EVs on an ImagestreamX MKII imaging flow cytometer

Joanne Lannigan[1], Luca Musante[2] and Uta Erdbruegger[2]

[1]School of Medicine, Flow Cytometry Core, University of Virginia, VA, USA; [2]Department of Medicine/Nephrology Division, University of Virginia, VA, USA

Introduction: It has been noted that scatter intensity of small particles is inversely proportional to the fourth power of the wavelength, indicating that more light is scattered at lower wavelengths than higher wavelengths. Using conventional flow cytometry, the use of violet lasers for scatter in analysing extracellular vesicles (EVs) has become more common. Imaging flow cytometry (ISX) is an important tool for characterising EVs. Traditionally, the ISX uses a far-red excitation source (758 nm) for side scatter. In this study, we explored whether the use of the 405 nm laser for side scatter would yield greater number of EVs detected by side scatter.
Methods: A variety of samples were used to assess the impact of laser wavelength on scatter detected by the 758 nm laser compared to the 405 nm laser. These included 180 nm silica particles, hUrine/plasma EVs (mean 225 nm), and 200 nm liposomes. All samples were acquired with each laser wavelength. Numbers of particles detected/mL and intensity of scatter signals were calculated and compared for each laser wavelength. Buffer alone was run to account for differences in background detection. These values were compared to the concentrations and sizes obtained by TRPS (qNano). In cases where samples contained fluorescence, these parameters were also assessed for concentration differences.
Results: In all cases, the number of particles detected by scatter with the 405 nm laser was significantly higher than with the 758 nm laser. This was also true of background detection in the buffer only sample, however, subtracting the additional background still led to higher numbers of detectable events with the 405 nm laser. In cases where samples were labelled with EV or lipid specific fluorochromes, the increased detection was shown to be specific particles of interest. The number of particles detected with the 405 nm laser was closer to the concentrations determined using the qNano. Scatter intensity values obtained with the 405 nm laser were also significantly higher than those obtained with the 758 nm laser, making them easier to distinguish from low level background particle detection.
Conclusion: Use of the 405 nm laser for scatter detection of EVs using the ISX imaging flow cytometer yields greater detection of EVs by scatter. The higher scatter intensity from this laser allowed for better separation from background signals.

PT05.14

Flow cytometers dedicated to the analysis of small particles: a powerful tool for EV characterisation

Jaco Botha, Mathilde Sanden, Morten Hjuler Nielsen and Aase Handberg

Department of Clinical Biochemistry, Aalborg University Hospital, Aalborg, Denmark

Introduction: Flow cytometry (FCM) is a widely used method for quantitation and characterisation of extracellular vesicles (EVs). Limitations with FCM has prevented detection of EVs below 200 nm corresponding to approximately a third of all EVs. Although flow cytometers specifically developed for measurement of small particles (SP-FCM) have been made available, they are scarce and their performance remain untested. Thus, we aimed to determine the sensitivity and resolution of SP-FCM. We further assessed the reproducibility of enumeration and characterisation of EVs in plasma.
Methods: Flow cytometry was performed on an Apogee A60 MicroPLUS flow cytometer. Sensitivity and resolution were assessed using 100 nm fluorescent silica beads and a cocktail of non-fluorescent silica beads ranging from 180–1300 nm, respectively. Reproducibility of measurements was assessed by measuring aliquots of silica beads (n = 42) and platelet-free plasma (PFP) from one healthy donor (n = 22) labelled with lactadherin-FITC, anti-CD41-APC and anti-CD36-PE. EVs were defined as phosphatidylserine-exposing (PS+) events <1000 nm. Concentrations were determined directly based on sample flow-rate and acquisition time without a bead control.
Results: We demonstrated that SP-FCM has sufficient sensitivity to discriminate 100 nm silica beads from background and resolution to discriminate silica beads ranging from 100–1300 nm from each other. In addition, EVs could be measured down to 100 nm, and we demonstrated that the bulk of EVs are within the 100–300 nm range, consistent with previous studies. Some variability was observed in concentration determination of silica beads (CV = 12.75–17.39%). However, concentration determination of EVs was reproducible (CV = 3.68–7.32%), as was median fluorescence intensities of EV phenotypes (CV = 1.44–6.63%).
Conclusion: This study demonstrates that SP-FCM is a powerful tool for EV characterisation with sufficient sensitivity and resolution to reproducibly measure the bulk of EVs present in bio-fluids. We believe that SP-FCM holds great potential for increasing our understanding of EVs in pathological conditions which could lead to discovery of sensitive, disease-specific biomarkers.

LBP.03

EVucation: Freely available interactive public engagement tools for scientists to communicate the role Extracellular Vesicles in the body and healthcare

Ryan C. Pink, Findlay R. Bewicke-Copley, Paschalia Pantazi, Bianca Paris, Priya Samuel and David RF. Carter

Oxford Brookes University, Oxford, United Kingdom

Introduction: It is widely understood throughout international Science and Technology that Public Engagement is required for healthy scientific research dialogue and a deeper public understanding of its importance. This engagement is fundamental in a growing field such as Extracellular

Vesicles (EVs), especially with its potential impact on the public through novel diagnosis and treatment.
Methods: Here we present a set of freely available interactive public engagement tools to provide an 'off-the-shelf' kit to help scientists communicate the diversity of EVs in the body and their beneficial role in healthcare. We have designed and tested these outreach tools:
Results:

- 'Exosome Monsters'– children decorate polystyrene balls for fridge-magnets while we explain to the child about cells and adults about EVs surface diversity (Key Stage 0, ages 3-5),
- 'Squishy Blood' – Using a body map with color-coded organs they put their hands in a blood bowl of slimy balls (that represent EVs) and have to find and sort them while we explain about the specifically loaded EV contents and how we can use that information in healthcare (Key Stage 1-2, ages 6-11).
- 'EV health scanner' – The audience pick a barcoded ball (blood EV) at random and scan it on a camera linked to a Raspberry pi running a pseudo diagnosis programme giving out a clinical report, while we talk about how EVs are used in diagnosis and therapy (Key stage 2+, ages 8 – adults).

Summary/Conclusion: These have been tested at various science festivals. The PDF instructions to recreate these along with downloadable Raspberry pi code and supporting posters are available from the group's website: www.carterlab.co.uk/Engagement/. We also propose to use this site as a two-way resource in which others are welcome to add their own public engagement ideas. The availability of such tools will help disseminate the excellent work being done by the EV community and will in
Funding: None

LBP.04

Identifying immune related miRNAs, studying the differences between erythrocyte and human rhinovirus infected HeLa cells derived microvesicles, a profiling using Firefly particle technology

Roberta F C. Freezor and Sheelagh Heugh

London Metropolitan University, London, United Kingdom

Introduction: Encapsulated in microvesicles (MV) are proteins and nucleic acids including miRNA molecules. The discovery of miRNAs added a new dimension to our understanding of complex gene regulatory networks, inter-cellular/organ communication, and now MV as miRNA transporters. The remarkable stability of extracellular miRNAs is interlinked with the formation of miRNA-vesicle packages; MV forms a protrusion then detaches from the cell surface translocating across the cell membrane, allowing miRNAs enter recipient cells and mediate cell-to-cell communication.
Methods: We investigated 68 miRNAs (immunology panel) in (**1**) erythrocyte, eMV control (**2**), eMV induced with $CaCl_2$ (**3**) eMV induced with $CaCl_2$ and human serum (**4**) in parallel with: HeLa cells (**5**), HeLa infected with Human Rhinovirus type 16 (**6**), HeLaMV Control (**7**), and HeLa infected with Rhinovirus type 16 MV (**8**). All MV samples were prepared using the classical ultracentrifugation method, miRNA samples were prepared using mirVana™miRNA Isolation Kit. miRNA concentration was measured by NanoDrop® ND-1000 UV-Vis (5µg) and processed by multiplex miRNA assay-Firefly particle technology (triplicates) and analysed with Firefly™Analysis Workbench software.
Results: 25 miRNAs out of 68 were expressed equally in all samples (excluding normalisers, negatives and haemolysis markers). *hsa-miR-10a, 30a-5p, 34a-5p, 132-3p, 196a-5p, 203a-3p, 210-3p, 422a, 181b-5p* and *744-5p* did not show expression in **1, 2,3**, and **4** samples, but was expressed in **5, 6, 7**, and **8** samples.*hsa-miR-223-3p* was not detected in **5,6,7** and **8** but strongly expressed **1, 2,3**, and **4** samples. *hsa-miR-146a-5p* and *150-5p* was not detected in **1, 5, 6,7** and **8** samples, but were slightly expressed in **2, 3**, and **4**. *hsa-miR-23a-3p* was not expressed in **1** but slightly expressed in **2, 3** and **4** and highly expressed in **5,6,7,8** samples. The hsa-miR- 16-2- 3p, 33a-5p, 125a-5p, 129-5p, 140-3p, 142-3p, 154-5p,155-5p, 200a-3p, 205-5p, 339-5p, 375, 376b-3p, 429, 431-3p and 523-5p did not show expression in the samples used here.
Summary/Conclusion: By analysing specific markers for each MV sample here, it can be suggested that our findings can positively contribute towards identifying MV involvement with; miRNA regulation, immunological, infective and intracellular actions.

LBP.05

Aptamer-based isolation of extracellular vesicles subpopulations: Finding the needle in a haystack

Sören Kuypers[1], Revathy Munuswamy[1], Jan D'Haen[2], Inge Nelissen[3], Joy I. Irobi[1], Baharak Hosseinkhani[1] and Luc Michiels[1]

[1]Hasselt University, Biomedical research institute, Martelarenlaan 42, 3500 Hasselt, Belgium; [2]IMO-IMOMEC, Hasselt University, Wetenschapspark 1, 3590, Diepenbeek, Belgium; [3]VITO NV, Boeretang 200, 2400 Mol, Belgium

Introduction: EV are considered as promising diagnostic targets, carrying valuable biomarkers for liquid biopsies. However, the downstream analysis of EV struggles with masking of disease specific information due to the vast majority of the EV coming from the homeostatic intercellular communication. Being able to isolate EV subsets while maintaining their functionality will increase their diagnostic potential. Therefore, our aim was to develop an aptamer based methodology to isolate potential intact disease involved EV subsets.
Methods: EV bulk was isolated from cells conditioned with TNF-α using SEC. The compatibility of the in-house developed monomeric C-reactive protein (mCRP) aptamer towards EV was confirmed using surface plasmon resonance (SPR). Next, a specific subset of EV was isolated using magnetic beads, covalently coated with aptamer. Release of the captured EV subset from the beads was confirmed using SPR, WB, NTA and TEM analyses. The integrity of the isolated EV was confirmed by monitoring the uptake of fluorescently labelled mCRP + EV subset into HUVEC.
Results: The EV bulk with a size range of about 100–200 nm was first isolated. SPR shows specific binding of EV under binding conditions and EV release was observed under non-binding conditions. Afterwards, the release of the EV subset was confirmed by different analyses. WB analysis showed the presence of classical EV markers such as CD63. Additionally, NTA and TEM verified that the EV subset was successfully isolated. The fluorescently labelled EV subset was taken up by HUVEC confirming that the EV isolated in this procedure are biologically intact.
Summary/Conclusion: This study shows that the proposed aptamer-based methodology can be used to successfully isolate intact EV subsets that are functionally active. This approach opens new ways to study the behavior of disease related EV subsets in target cells.
Funding: This work was financed by Hasselt University and by EFRO through the Interreg V Grensregio Vlaanderen Nederland project Trans Tech Diagnostics.

LBP.06

Free flow electrophoresis allows preparation of EV fractions with high recovery and purity rates

Gerhard Weber[1], Robert Wildgruber[1], Simon Staubach[2], Robin Dittrich[3], Peter Horn[3], Verena Boerger[4] and Bernd Giebel[2]

[1]FFE Service; [2]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden; [3]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, German; [4]Institute for Transfusion Medicine, University Hospital Essen, University Duisburg-Essen, Essen, Germany

Introduction: Currently, it remains a challenge to prepare extracellular vesicles (EVs), especially those from body fluids, such as plasma, to high purity. Neither fractionation by density nor by size is sufficient to separate EVs from all contaminants e.g. high and low density lipoprotein (HDL/LDL) and other contaminating components. For now, a time-consuming combination of two methods (density and size) is required to enrich EVs to high purities, regularly resulting in low EV recoveries. Free Flow Electrophoresis is a well-established preparative and micro-preparative method to separate analytes with inherent difference of charge density and/or difference of pI-value.
Methods: Free Flow Interval Zone Electrophoresis (FF-IZE), using media of different pH-values, ranging from pH = 8 to pH = 4.8 offers most suitable protocols for the quantitative separation of amphoteric analytes,

like proteins and peptides from non-amphoteric analytes like lipid vesicles, DNA and RNA.
Results: Within our ongoing project we have optimized FF-IZE-pH protocols for the purification and isolation of EVs as well DNA and RNA from cell culture supernatants and human plasma samples. Upon screening for EV-specific samples in a dot blot system, EV-specific antigens are specifically recovered in a selected number of fractions. Currently, we characterize the identified fractions in more detail. For the enumeration of prepared EVs we use the Nanoparticle Tracking Analysis (NTA). Furthermore, the presence of EV markers and the absence of contaminants are analyzed by Western Blot. We document the appearance of isolated EVs by transmission electron microscopy and determine the miRNA profiles of the obtained fractions.
Summary/Conclusion: The principle of FFE, the EV isolation strategy and our ongoing results will be presented.

LBP.07

Visualization of extracellular vesicles derived from human bone marrow mesenchymal stem cells using fluorescent and magnetic labels; *in vitro* and *in vivo* studies

Sylwia Koniusz[1], Anna Andrzejewska[1], Andrea Del Fattore[2], Elżbieta Karnas[3], Malgorzata Frontczak-Baniewicz[4], Hanna Kozlowska[5], Maurizio Muraca[6], Miroslaw Janowski[7] and Barbara Lukomska[1]

[1]NeuroRepair Department, Mossakowski Medical Research Centre, PAS, Warsaw, Poland; [2]Multifactorial Disease and Complex Phenotype Research Area, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy; [3]Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; Malopolska Centre of Biotechnology, Krakow, Poland; [4]Electron Microscopy Platform, Mossakowski Medical Research Centre, PAS, Warsaw, Poland; [5]Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Centre, PAS, Warsaw, Poland; [6]Department of Women's and Children's Health, University of Padua, Padua, Italy; [7]NeuroRepair Department, Mossakowski Medical Research Centre, PAS, Warsaw, Poland; Russel H. Morgan Department of Radiology and Radiological Science, Division of MR Research, The Johns Hopkins University School of Medicine, Baltimore, USA

Introduction: Mesenchymal stem cells (MSCs) have shown both anti-inflammatory and pro-regenerative activity in a variety of disorders. Recent studies support the notion that the signals responsible for these therapeutic effects are at least partially conveyed by extracellular vesicles (EVs). Despite growing interest in EVs as therapeutic tools, little information is available on the fate of these nanoparticles following *in vivo* administration because of methodological hurdles. *The aim of the study* was to optimize the method of EVs visualization for *in vitro* and *in vivo* biodistribution studies.
Methods: The experiments were performed using human bone marrow mesenchymal stem cells (hBM-MSCs) (Lonza). hBM-MSCs were labelled with PKH26 (Sigma) and iron nanoparticles conjugated with rhodamine (Molday, BioPAL) and co-stained with anti-CD9, -CD63 and -CD81 (tetraspanins) and MSCs antibodies. EVs were isolated from the culture media of previously labelled hBM-MSCs. The size, number, morphology and biomarker expression of hBM-MSC-EVs were identified by Nanosight analysis, high-resolution flow cytometry, transmission electron microscopy, superresolution illumination microscopy and MRI. The i*n vivo* studies were performed in adult male Wistar rats with focal brain injury of 1µl/50nmol ouabain injection into the right hemisphere. Two days after the brain insult1.3x10^{9}hBM-MSC-EVs labelled with Molday or stained with PKH26 were infused into the right internal carotid artery and analysed in rat brain immunohistochemically using confocal microscopy.
Results: *In vitro* studies revealed the presence of intracellular vesicles positively stained with Molday ION or PKH26 visible inside hBM-MSCs co-expressed CD44, CD73, CD90, CD9, CD63 and CD81 markers. The isolated EVs represented heterogeneous population of various size (50-300 nm) and kept their markers after isolation. hBM-MSC-EVs transplanted intraarterially in focal brain injured rats migrated into the right hemisphere near the ischemic injury.
Summary/Conclusion: PKH26 and Molday ION enable to visualize hBM-MSC-EVs *in vitro* and *in vivo* after their intra-arterial transplantation. Molday ION tagging may allow additional imaging of EVs delivery using MRI.

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LBP.08

MicroRNA biogenesis and heterogeneous miRNA distribution in cancer EVs

Nils J. Groenewegen, Catrin Lutz, Alba M. Losada, Monique A.J. van Eijndhoven and D. Michiel Pegtel

Exosomes Research Group, Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

Introduction: It is now firmly established that mature 22nt miRNAs are detected in populations of extracellular vesicles (EVs) and exosomes. Exosomal miRNAs have physiological effects in recipient cells but the question remains whether they can non-cell autonomously modulate gene expression. Presumably, loading of a single guide strand of miRNAs into RISC (a prerequisite for active repression of mRNA translation) is inefficient compared to loading of miRNA duplexes that are typically not found in exosomes. Possibly a chaperone system exists that can catalyze this process, but such evidence is lacking. One study described that cancer cells secrete a subtype of EVs, that support cell-independent but Dicer-dependent miRNA biogenesis as an essential feature of their reported protumorigenic potential in mice (Melo et al., Cancer Cell 2014).
Methods: We used RT-PCR to measure miRNA levels in cancer EVs purified by standard ultracentrifugation protocol and in addition also in EVs purified by Size-exclusion chromatography (SEC), including serial dilutions of SEC-purified EVs to reveal miRNA distribution amongst EV subpopulations.
Results: We repeated these experiments but were unable to measure significant miRNA biogenesis in cancer EVs purified by standard ultracentrifugation protocol. We reasoned that abundant protein-complex bound miRNAs might obscure results and calculations. We next set out to recapitulate the process of cell-independent miRNA biogenesis in SEC-purified cancer EVs from cancer cell-lines and bio-fluids from cancer patients. Our results suggest that cell-independent miRNA biogenesis in SEC-purified EVs is very inefficient or occurs only in a very small sub population of vesicle such as large oncosomes or apoptotic bodies. However using limiting dilution analysis we could not find evidence for miRNA biogenesis in a small subpopulation of EVs, agreeing with stoichiometry calculations suggesting that EV populations carry less than 1 mature miRNA copy of a single species (Chevillet et al., PNAS 2014).
Summary/Conclusion: Our observations rule out pervasive miRNA-biogenesis in SEC-purified EVs and disfavor miRNA biogenesis in small EV subpopulations. Studies are ongoing, aimed at defining cancer EV subpopulations with distinctive RNA content.
Funding: Cancer Center Amsterdam Foundation (CCA-2016)

LBP.09

Analysis of coat and whole proteins from exosomes using MS compatible surfactants

Ayako Kurimoto and Tatsutoshi Inuzuka

Fundamental Research Department, FUJIREBIO Inc.

Introduction: Exosomes are a type of extracellular vesicles secreted from all types of cells via endosomal pathway and found in most body fluids, including blood, urine, saliva, blood, breast milk, and cerebrospinal fluid. Many biologically active molecules such as protein, mRNA, miRNA, DNA and phospholipid are found in exosomes. Exosomes have been suggested to mediate cell-to-cell communication via proteins e.g. integrins, and to be associated with various disease conditions. In order to explore the function of exosomes, highly efficient, comprehensive proteomic analysis is essential. To this end, surfactants are generally used to enhance protein digestion efficiency, which results in the increased total sequence coverage and number of identified peptides and proteins in LC-MS.
In this study, we compared the efficiency of commercially available surfactants using cancer cell conditioned medium. We have also assessed the presence of cancer marker within the exosomes.

Methods: Exosomes are collected from hepatocellular carcinoma HepG2 conditioned medium by ultracentrifugation, and lysed using commercially available MS-compatible acid-labile surfactants (e.g., AALS, and NALS) before being digested by proteases. Obtained peptides were analyzed using Triple TOF5600+ system and ProteinPilot software. A tumor marker, carcinoembryonic antigen (CEA) contained in the exosomes from pancreatic cancer cell line AsPC-1 was quantified using immunoassay analyzer.
Results: HepG2 conditioned medium has increased by the addition of lysis step using various kinds of MS-compatible surfactants compared to guanidine-HCl treatment, with the exception of AALS II. Immunoassay analysis revealed that CEA in exosomes from AsPC-1 has increased by the solubilization treatment using detergents, except for AALS II as well. These results suggest that AALS II detergent may be beneficial for identifying coat proteins on the surface of exosomes from HepG2.
Summary/Conclusion: Addition of solubilization step using detergents for proteomic analysis has increased the number of identified proteins from exosomes. However, AALS II treatment has resulted in the reduction of identified protein number, as well as the amount of CEA detected. AALS II surfactant may be applicable to identify the outer coat proteins of exosomes from HepG2.

LBP.10

Nanocellulose filters for extracellular vesicle purification
Prateek Singh[1], Jonne Ukkola[2], Henrikki Liimatainen[2] and Seppo Vainio[1]

[1]University of Oulu, Finland; [2]Fibre and Particle Engineering, University of Oulu, Finland

Introduction: Extracellular vesicle purification is key in deducing the precise function of the EVs in biological processes. Here we have developed a nanocellulose based EV filter which allows specific capture of EVs from solution. Nanocellulose-based materials are based on long, polymeric cellulose chains consisting of hundreds to several thousand repeating glucopyranose units forming strong and stable crystalline regions and more flexible amorphous regions. Cellulose can be found in various fungi, algae or bacterial sources, but the most of the nanocellulosic materials are produced from wood- or plant-derived sources via mechanical disintegration techniques coupled with chemical or biological pretreatment methods. Nanocellulose refers to cellulose particles with at least one dimension in nanoscale (1-100 nm) and it can be divided into two main categories: cellulose nanocrystals (CNC) and cellulose nanofibrils (CNF).
Methods: EVs from RENCA, LNCaP cell lines were used to assess the performance of the nanocellulose filters. Cellulose nanofibrils with varying surface groups were prepared. Three different qualities of cellulose nanofibrils (deep eutectic solvent (DES) CNF, aldehyde functionalized DADES CNF and dicarboxylic cellulose nanofibrils (DCDES NFC)) were prepared from bleached birch (Betula verrucosa and pendula) chemical wood pulp obtained in dry sheets.
Results: Three different nanocellulose filters were prepared and used to pull down EVs from dilute solutions. In our preliminary tests, bare, non-functionalized nanocellulose is neutral towards EVs. Carboxyl –modified nanocellulose on the other hand, showed preferred binding to the EVs. BCA protein assay and transmission electron microscopy were utilized to verify EV filtration.
Summary/Conclusion: The nanocellulosic filters were rapid alternative to EV purifications as compared to lengthy ultracentrifugation. Antibody functionalized nanocellulose filters can offer specific EV capture from

LBP.11

Porous nanomaterials for exosome capture and in situ processing
Wenwan Zhong[1] and Xiaoni Fang[2]

[1]Department of Chemistry; [2]University of California, Riverside, CA, USA

Introduction: The exosome-derived analytes including RNA, DNA, proteins, metabolites and lipids may mirror the altered state of the cell of origin. Therefore, profiling exosomal contents can help to identify valuable markers for better understanding of the function and origin of exosomes in the circulating system. However, Exosomes are only 30-100 nm in diameter, and the total amounts of the enclosed biomolecules are small. Thus, exosome analysis always starts with exosome enrichment from biological fluids. Isolations are typically based on their size and density using ultracentrifugation, or with microfluidic devices; but these methods cannot completely remove other lipid-structures like the high- or low-density lipoprotein complexes, and downstream analysis remains challenging due to the membrane structures.
Methods: Herein, we propose a new approach that combines efficient isolation of exosomes enabled by porous nanomaterials with in situ sample processing for rapid profiling of exosomal proteins. The uniform pore structures (about 100 nm size) of the graphene forms can trap the exosomes while excluding the large microvesicles (> 100 nm). Specific exosome recognition can also be obtained by antibodies targeting exosome's surface markers. Moreover, in situ protein digestion can be achieved within the porous structures and the peptides can be purified easily.
Results: We proved that our material could trap the polystyrene beads with sizes ranging from 50-200 nm, while the ones with larger sizes were excluded. The enrichment took less than 30 minutes, followed by rapid protease digestion. The high surface-area-to-volume ratio and significantly improved the total number of proteins identified. To further improve the proportion of membrane protein identification, we did the second enrichment step employing the unmodified graphene form to adsorb the membranous peptides via after in situ protease digestion, and > 60% of the identified peptides were membrane peptides.
Summary/Conclusion: We report a new method that utilizes porous nanoamterials to enhance content analysis of exosomes. We expect our method can help to identify more surface markers for exosomes and contribute to the functional study of exosomes and other extracellular vesicles.
Funding: R01CA188991

LBP.12

A dielectrophoretic nanopore device with spatiotemporal resolution for microvesicles entrapment and quantification near living cells
Leilei Esfandiari, Ankit Esfandiari and Leyla Esfandiar

University of Cincinnati, OH, USA

Introduction: Exosomes are small membrane vesicles, ~100nm in diameter, secreted by cells and can be found in body fluids. They play important roles as molecular cargoes to deliver gene regulating microRNAs and key proteins between cells and thus, they have become a molecule of interest to many researchers as a circulating diagnostic and prognostic biomarker. The major challenge associated with exosomes has been the effective and selective isolation and quantification. Currently, tedious and time-consuming ultracentrifugation steps combined with filtration techniques have been utilized for separation and purification of these vesicles from cell culture medium or body fluids. In this work, we have developed a new low-voltage, ultra-sensitive, and rapid DC dielectrophoretic (DEP) nanopipette tool with the capability to isolate and quantify biomolecules based on their surface charge and size.
Methods: A borosilicate nanopipette with 500nm diameter was back filled with electrolyte solution and was inserted into two electrically neutral reservoirs by means of a PDMS chamber structure. Nanoparticles and artificial liposomes with various surface charge density and diameters were used as model system for the proof of concept experiments. The particles with different concentration were injected in to the chamber in front of the pipette's tip and a DC potential was applied across the nanopipette. Upon the applied potential, the ionic current across the pipette was measured and the movement of particles was recorded microscopically.
Results: The correlation between the trapping efficiency and electric field strength, salt concentration in buffer, particle type and diameter, pore size was studied empirically and compared with simulation results. A mixture of nanoparticles and liposomes with different diameters were selectively trapped at the tip of the pipette. Upon entrapment, the unique conductance change across the pore was measured which indicated the quantitative detection of the specific molecule.
Summary/Conclusion: This novel nanopore-DEP device can isolate the target molecules with DC voltage as low as 0.6V/Cm in a buffer with a

high ionic concentration in less than five minutes. Also, this device has a high special resolution and thus has a potential to entrap secreted biomolecules including exosomes near living cells.
Funding: University of Cincinnati Startup Fund

LBP.13

Two-dimensional electrophoresis-based proteomic analysis for urinary extracellular vesicles

Aki Nakayama Howley, Hideka Shigeta and Shiro Iijima

Bunkyo Gakuin University, Tokyo, Japan

Introduction: Extracellular vesicles (EVs) produced form renal epithelial cells are increasing in interest the last 5 years. The main problem encountered during purification of urinary EVs is co-precipitated Tamm Horsfall protein (THP), which is the most abundant protein in urine of healthy subjects secreted from the thick ascending limb of Henle's loop. We previously reported that the PVDF membrane filtration was an easy and effective method for removing co-precipitated THP. Two-dimensional polyacrylamide gel electrophoresis (2DE) was also useful for proteomic analysis of urinary EVs because the isoelectric point of THP is about 3.5 and the other majority of protein spots are isolated from it. Using this method, in the present study, we developed a protein map of urinary EVs.
Methods: Urinary EVs were isolated from a pooled urine sample of healthy subjects by differential ultracentrifugation. PVDF membrane filtration was performed after ultracentrifugation at 200,000g. Urinary EVs were characterized by immune electron microscopy, Western blot and flow cytometry. Isolated EVs were analyzed by 2DE Protein spots were subsequently trypsin-digested and analyzed by liquid chromatography-tandem mass spectrometry.
Results: Immune electron microscopy verified the presence of urinary EVs. The mean diameter of urinary EVs was 42.1 ± 13.9 nm. Eighty-nine proteins were identified from protein spots on 2DE by proteome analysis and classified using Gene Ontology that 44% were cytoskeleton and membrane 15% were cytosol, and 10% were endocytosis related proteins. A functional biomarker of tubular stress of tropomyosin alpha-4 chain was found in this protocol.
Summary/Conclusion: We have developed a protein map of urinary EVs by using 2DE. Urinary EVs contain renal specific markers and 2DE-based analysis was useful and effective for identification of candidate biomarker proteins. These results contribute to clarifying the functional characteristics of urinary EVs.

LBP.14

Membrane markers profiling: Comparative analysis of microvesicles derived from erythrocyte and HeLa cells infected with Human Rhinovirus type 16

Roberta F. C. Freezor and Sheelagh Heugh

London Metropolitan University, London, United Kingdom

Introduction: The detection and profiling of markers on microvesicles (MV) is important in the context of developing a potential tool for early diagnosis of diseases and profiling surface proteins can contributes towards the understand of MV acting as a form of long-distance cell-cell communicator.
Methods: In order to determine the expression of surface markers, MV samples including (**1**) erythrocyte MV (eMV) control, (**2**) eMV induced with $CaCl_2$ (**3**) and eMV induced with $CaCl_2$ and human serum, HeLa cells MV Control (**4**) and Hela cells infected with Human Rhinovirus (HRV) type 16 MV (**5**) were labelled with a variety of cluster differentiation(CD) FITC-Conjugated antibodies through the direct method and analysed by Flow cytometry Guava Express Plus software along with the Sub-micron Particle Size Reference Kit (side scatter signals against green scatter signals of reference microspheres sizes 0.5 to 2.0μm) acting as a template for fluorescence intensity using the ExpressPlus software.
Results: Annexin V (+VE) and IgG (-VE) were important and relevant parameters (controls) considered to ensure that only MV was detected, this was also used to ensure the correct gate was created (fluorescent and size). Signals from erythrocyte markers (CD235ab) were clearly +VE on eMV >93%, and it was highly -VE for **4** and **5** samples >91%. CD54 (HRV marker) showed >78% +VE for **4** and >96% for **5** but >78% -VE for all eMV samples. CD46 was >66% -VE in eMV samples and >92% +VE in **4** and **5** samples. Moreover, MV samples did not bind to CD14 demonstrating that eMV samples were only derived from erythrocyte cells and were not contaminated with any other blood cells type, it also showed -VE staining in **4** and **5**. CD58 and CD36 were expressed in all samples, in contrast to CD63 that was not expressed in eMV control but slightly expressed in **4** and **5** (>66%). Whereas, HLA-ABC was >55% negative in all eMV samples but highly expressed in **4** and **5** samples (>91%).
Summary/Conclusion: The selected panel of CD expression including known (-VE) and (+VE) markers revealed that MV express the same antigenic markers as those present in the parent cell. The groups of MV populations did not have a huge significance of expression within itself, being the same level of expression for almost all samples (each label) for the majority of the CD chosen here.

LBP.15

Lipidomic analysis of extracellular vesicles derived from propionibacterium acnes

Jin Her[1], Jinseong Jeon[1], Sangeon Shin[2] and Changill Ban[1]

[1]Pohang University of Science and Technology, Pohang, Republic of Korea; [2]POSTECH

Introduction: Propionibacterium acnes is an anaerobic normal flora, mainly found in the skin and gastrointestinal tract. Recently, the pathophysiological effects of P. acnes not only in acne progression but in various diseases has been reviewed. As an emerging mode of communication in bacteria, extracellular vesicle (EV) has been reported to conduct critical pathophysiological functions.
Methods: For the comprehensive understanding of the lipidomic profiles of P. acnes, we report comparative lipidomic analysis of P. acnes and P. acnes EV for the first time and identified 290 vesicular lipids with high confidence using triplicate LC-MS/MS analyses.
Results: In this research, we suppose that P. acnes EV might conduct distinguishing functions in micro-environments for the distinct pathogenicity and lifestyle of P. acnes.
Summary/Conclusion: We expect these findings to provide useful clues for understanding biological and pathophysiological mechanisms of P. acnes and for clinical applications such as vaccine development, diagnostics and therapeutics.

Poster Session PT06 – Non-Cancer EV Biomarkers
Chairs: Luca Masante and Julie Saugstad 5:15–6:30 p.m.

PT06.01

Specific types of miRNAs from urinary extracellular vesicles identify pathogenesis of kidney stones and Randall's plaque in humans

Muthuvel Jayachandran[1], Xiangling Wang[2], Robin Chirackal[3], John Knoedler[3], Amy Krambeck[2], Felicity Enders[3], Andrew Rule[3], Pritha Chanana[3] and John Lieske[3]

[1]Mayo Clinic College of Medicine, MN, USA; [2]Mayo Clinic, MN, USA; [3]Mayo Clinic Rochester, MN, USA

Introduction: Micro RNAs (miRNAs) regulate various cellular processes through modification of post-transcription and translation of target mRNAs. The role of miRNAs in the pathogenesis of kidney stones and its likely precursor Randall's plaque (RP) is not known. This study was designed to compare specific miRNAs within urinary extracellular vesicles (EVs) between stone formers with varying disease severity and controls.
Methods: Bio-banked cells-free urine samples from kidney stone formers with low plaque (LP, $n = 4$, <5% papillary surface area coverage) and high plaque (HP, $n = 4$, >5% papillary surface area coverage), first time stone formers ($n = 4$) and non-stone forming controls ($n = 4$) were used in this study. Urinary EVs were isolated by ExoQuicK^{Tc} and miRNAs within EVs were quantitated by XRNA Exosome RNA-Seq Library Kit (System Biosciences, Palo Alto, CA). Differentially expressed miRNAs between HP and LP stone formers, and between first time stone formers and controls with a p-value of 0.05 or lower were selected for pathway analysis.
Results: A group of miRNAs that contribute to calcification, cell proliferation, acute kidney injury, renal fibrosis, pro-apoptotic and pro-inflammatory processes including miR-223-5p, miR-199a-3p, miR-664a-3p, miR-489-3p, miR-26b-3p, miR-146b-5p, miR-148b-3p, miR-1299 and miR-24-2-5p were increased 6 to10- fold, whereas miRNAs that contribute to anti-apoptotic and anti-inflammatory processes, prevent renal fibrosis, ischemic injury, and chronic kidney disease such as miR-499a-5p, miR-455-3p, miR-483-5p, miR-6087 and miR-532-3p were decreased 2 to 5-fold in first time stone formers compared to controls. MiR-489-3p and miR-146b-5p were increased 5.8–6.6 fold whereas miR-483-5p, miR532-3p and miR-6087 were decreased 4.5–7 fold in low-RP compared to high-RP stone formers.
Conclusion: These specific miRNAs in EVs may provide new insights into early renal cellular pathogenesis in kidney stone and RP formation, and new tools for the screening, diagnosis, and risk stratification of persons with calcium stone disease.

PT06.02

Proteomic identification of exosomal VDAC1: a potential urinary biomarker for detecting early renal fibrosis

Dekun Wang, Chuanai Chen, Zhujun Zhang and Xiaoyue Tan

The Medical School of Nankai University, Nankai, China

Introduction: Non-invasive tools for evaluation of early renal fibrosis are of great value for either detecting the kidney fibrotic lesion or predicting the prognosis and therapeutic reaction.In this study, we aimed to identify the fibrosis related biomarkers in the urinary exosomes via proteomic screening of the exosomes in the legumain knockout mice.
Methods and Results: Firstly, we set up a novel age-related mouse model of kidney fibrosis via genomic knockout of legumain, a conseverd asparaginyl endopeptidase physiologically expressed at renal tubuli. Level of renal fibrosis was evaluated via hydroxyproline assay and masson-trichrome staining. Legumain knockout mice showed significant renal fibrosis beginning at 3 months old with normal serum creatinine value. We isolated urine exosomes of 2 months old mice by ultracentrifugation and authenticated them by electron microscopy and western blot. Exosomal proteins were then separated by 1-D SDS-PAGE and the differentially expressed bands between 25 and 35 kDa were cut-off from the gel. Through LC-MS/MS analysis, Voltage dependent anion channel 1 (VDAC1) was identified from 45 candidate proteins and the differential expression of Vdac1 was further validated in urinary exosomes by Western blot and PCR. Via immunofluorescence study, we verified that expresssion of Vac1 was located on the outer membrane of mitochondria and mainly at renal tubuli. Then we examed exsomal vdac1 level via western blotting in the fibrosis mouse model at different ages. Our data showed exosomal vdac1 volume correlated positively with fibrosis level in the fibrotic kidney assessed by thehydroxyproline assay, as well as the activity of TGF-β measured by ELISA. In the human urine exosome samples, Vdac1 expression was high in the patients with renal fibrotic diseases compared with the normal control. *In vitro*, Vdac1 could be identified in the exsomes isolated from cultured renal tubular cells. And it could be induced by different pro-fibrotic stimuli, such as TGF-β1 and aristolochic acids in the culture renal tubular epithelial cells.
Conclusion: In this study, we identified Vdac1 in the urine exosomes as an potential index to evaluate early stage of renal fibrosis.

PT06.03

Urinary extracellular vesicles carrying markers of kidney injury and renal stem cells differ between women and men and with age in living kidney donors

Muthuvel Jayachandran[1], Rangit Vallapureddy[2], Aleksandar Denic[2], Virginia Miller[2], John Lieske[3] and Andrew Rule[3]

[1]Mayo Clinic College of Medicine, MN, USA; [2]Mayo Clinic, MN, USA; [3]Mayo Clinic Rochester, MN, USA

Introduction: The prevalence of kidney disease increases with age and is higher in men than in women. Injured or activated renal cells release extracellular vesicles (EVs) that could reflect ongoing renal pathophysiology.
Methods: This study was approved by Mayo Clinic Institutional Review Board. Bio-banked cells-free random urine from living healthy kidney donors aged from 20 to 70 years old was studied. Urinary EVs >0.2 micron were analysed by an established digital flow cytometry method and appropriate antibodies. EV counts were calculated as EV/μL urine and normalised to EV/mg creatinine. Ratios of EV/CD63 (exosome) or EV/annexin-V (microvesicle) were also calculated for data analyses.
Results: Median age (47 and 44 years) and glomerular filtration rate (GFR, 101 and 102 ml/min/1.73 m^2) were similar between women ($n = 88$) and men ($n = 54$). Urinary EVs positive for renal injury markers (beta-2 microglobulin (beta-2M), cystatin C, laminin alpha-5 (LAMA5), and neutrophil gelatinase-associated lipocalin (NGAL)) were greatergreater ($p < 0.05$) in women than men. Glomerular (CD90)- and tubular (CD133)-stem/progenitor cell-derived EVs did not differ by sex. Urinary EVs positive for beta-2M, cystatin C, LAMA5 decreased ($p < 0.05$) whereas tubular stem/progenitor cell-derived EVs increased ($p < 0.05$) with age. EVs positive for LAMA5 positively ($p < 0.05$) but EVs positive for CD133 negatively ($p < 0.05$) correlated with GFR. Tubular stem/progenitor-derived EVs increased ($p < 0.05$) with percentage of globally sclerotic glomeruli observed in kidney biopsy obtained before transplantation.
Conclusion: These results demonstrate that among apparently healthy living adults there are sex-and age-associated differences in urinary EVs bearing renal injury markers. Results may also provide clues regarding mechanisms of sex differences in the prevalence of kidney disease. LAMA5 and CD133 positive EVs merit further study as potential biomarkers of kidney function among healthy and diseased patients.

PT06.04

Specific types of urinary extracellular vesicles differentiate type 1 primary hyperoxaluria patients without and with nephrocalcinosis or kidney stones

Stanislav Yuzhakov[1], Neil Sha[1], John Lieske[2], Andrew Rule[2], Dawn Milliner[1] and Muthuvel Jayachandran[3]

[1]Mayo Clinic, MN, USA; [2]Mayo Clinic Rochester, MN, USA; [3]Mayo Clinic College of Medicine, MN, USA

Background: Primary hyperoxaluria type 1 (PH1) is an autosomal recessive disorder characterised by hepatic overproduction of oxalate. The resulting hyperoxaluria can cause nephrocalcinosis and calcium oxalate stones. Kidney cell-derived extracellular vesicles (EVs) could reflect these pathophysiologic processes.
Methods: Bio-banked (cells-free) urine from 15–30 years old male and female PH1 patients without (n = 10) and with nephrocalcinosis (n = 6) or stones (n = 9) and age-/sex-matched (± 5 years) living kidney donors (n = 25) was studied. Urinary EVs (>0.2 μm) were analysed by digital flow cytometry and analysed as EVs/μL urine and EVs/mg urine creatinine.
Results: PH1 patients had significantly (PConclusion: Distinct urinary EV populations were observed among PH1 patients without and with nephrocalcinosis or stones. These results suggest that EVs released from specific populations of renal cells of PH1 patients reflect nephrocalcinosis or stone status, and further study of urinary EVs may allow identification of novel biomarkers and provide clues regarding the pathogenesis of renal calcification in this disease.

PT06.05

Characterisation and mechanism of increased exosomal CYP2E1 and other P450 isoforms in alcoholic patients and alcohol-exposed rodents

Young-Eun Cho[1], Esteban Mezey[2], James P. Hardwick[3], Norman Salem Jr[1] and Byoung-Joon Song[1]

[1]Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, NIH; [2]Department of Medicine, The Johns Hopkins University School of Medicine, MD, USA; [3]Department of Integrative Medical Sciences, College of Medicine, Northeast Ohio Medical University, OH, USA

Introduction: The ethanol-inducible cytochrome P450-2E1 (CYP2E1) and P450 isoforms are involved in the metabolism of many substrates with the production of metabolites and reactive oxygen species in the liver and other tissues. We hypothesised that elevated amounts of CYP2E1 and other P450 isoforms can be secreted into circulating exosomes in alcohol-exposed rodents and alcoholic patients in a CYP2E1-dependent manner. Thus, this study was aimed to investigate the role of CYP2E1 in increasing the levels of CYP2E1 and other P450 proteins in plasma exosomes from alcohol-exposed rodents and human alcoholics.
Methods: Female Fischer rats and wild-type (WT) or *Cyp2e1*-null mice were exposed to three oral doses of ethanol (4–6 g/kg/dose) or dextrose (as control) at 12 h intervals and sacrificed at 1 h after the last ethanol dose. Plasma exosomes from alcohol-exposed rodents, alcoholics and their respective controls were isolated and confirmed by immunoblots for exosomal marker proteins and size measurements. The exosomal proteins were characterised by immunoblot analyses.
Results: The amounts of exosomes and exosomal CYP2E1, CYP2A6, CYP4B proteins were markedly elevated in alcoholics and alcohol-exposed rats and mice, which exhibited hepatic steatosis, than the respective controls. The elevated amounts of exosomes and exosomal P450 proteins were significantly reduced in ethanol-exposed rats fed a diet containing n-3 polyunsaturated fatty acids. Further, the increased number of exosomes and the exosomal CYP2E1 and P450 isoforms in alcohol-exposed WT mice were significantly blunted by co-treatment with a CYP2E1 inhibitor chlormethiazole or an antioxidant *N*-acetylcysteine or in the ethanol-exposed *Cyp2e1*-null mice.
Conclusion: These results suggest the role of CYP2E1 and oxidative stress in promoting the ethanol-mediated secretion of exosomal proteins. Additionally, exosomal CYP2E1 could be used as a potential biomarker for alcohol exposure and/or alcohol-induced fatty liver.

PT06.06

Hepatocyte-derived exosome enrichment and cell culture methods optimisation for the identification of novel DILI biomarkers

Sarah Thacker[1], Manisha Nautiyal[1], Natalie Holman[2], Monicah Otieno[3], Paul Watkins[1] and Merrie Mosedale[1]

[1]UNC Chapel Hill Eshelman School of Pharmacy, NC, USA; [2]UNC Chapel Pharmacy, NC, USA; [3]Janssen Research and Development, LLC

Introduction: We have previously demonstrated that hepatotoxicants induce alterations in hepatocyte-derived exosomes (HDE) prior to overt necrosis, supporting a role for HDE in the pathogenesis of drug-induced liver injury (DILI). Because HDE contain liver-specific mRNAs, miRNAs, and proteins, they may have value as sensitive and specific biomarkers of DILI. In order to explore the DILI biomarker potential of HDE, the objectives of this study were to (1) identify the best method for enrichment and (2) optimise cell culture methods to compare the number and content of HDE released from primary human hepatocytes (PHH) in response to DILI compounds.
Methods: To evaluate exosome enrichment, vesicles were isolated from the culture medium of HepG2 cells using ultracentrifugation (UC), OptiPrep density gradient ultracentrifugation (ODG), and ExoQuick-TC™ (EQ). To evaluate the effect of a Matrigel® overlay on exosome release, exosomes were enriched from the culture medium of HepaRG cells using UC. Nanoparticle tracking analysis was performed to assess vesicle number and size. Total RNA extracted from vesicles was used to determine the quantity (Quant-iT™ *RiboGreen*®) and fraction of miRNA that was vesicular vs. AGO2 bound (immunoprecipitation). Total protein was quantified and exosomal protein enrichment was evaluated via Western blotting.
Results: EQ resulted in a significantly higher number of exosome-sized particles than UC ($p < 0.001$) or ODG ($p < 0.0001$). Particle size and variation using UC and EQ were similar (~100 ± 10 nm), however ODG enriched for particles significantly larger in size ($p < 0.05$). EQ and UC resulted in comparable levels of vesicular RNA and protein, however UC had significantly more vesicular RNA and CD63 protein when compared to EQ or ODG ($p < 0.05$). No significant differences in particle number were observed across Matrigel concentrations ranging from 0–0.25 mg/mL.
Conclusion: These data suggest that both UC and EQ enrichment result in significantly more HDE than ODG, but UC produces a purer population of HDE. Matrigel overlay does not inhibit the release of HDE. We conclude that UC-based enrichment provides the optimal combination of HDE quantity and purity and Matrigel overlay can be used in PHH culture for the identification of novel exosome-based biomarkers for DILI.

PT06.07

Elevations in circulating extracellular vesicle miR-21 as a biomarker of developing Type 1 diabetes mellitus

Alexander Lakhter[1], Farooq Syed[2], Bernhard Maier[2], Raghavendra Mirmira[1], Carmella Evans-Molina[3] and Emily Sims[1]

[1]Department of Pediatrics, Section of Endocrinology and Diabetology, Center for Diabetes and Metabolic Diseases, IU School of Medicine; [2]Center for Diabetes and Metabolic Diseases, IU School of Medicine; [3]Department of Pediatrics, Section of Endocrinology and Diabetology, Department of Cellular and Integrated Physiology, Center for Diabetes and Metabolic Diseases, IU School of Medicine

Type 1 diabetes (T1D) develops over time, such that by the time of typical diagnosis, patients have already lost 80% of their pancreatic beta cell mass. Strategies for detection of T1D, prior to widespread loss of the β cells, are acutely needed for improved outcomes of preventative interventions. MicroRNAs (miRNAs) released in extracellular vesicles (EVs) have been proposed as ideal biomarkers due to their stability and feasibility of detection. Previous work from our lab demonstrated that β cell miR-21 production is induced by inflammation, and RT-qPCR analysis of diabetic NOD mouse islets revealed a ~4-fold increase in miR-21 expression compared to NOR controls. We hypothesised that the inflammatory milieu of developing T1D may also increase miR-21 in β cell EV cargo. EVs released by INS-1 β cells exposed to a cytokine mix of IL-1β, INFγ and TNFα were isolated using ExoQuick reagent. RT-qPCR revealed an 8-fold increase in EV miR-21. Similarly, a 5-fold increase in miR-21 content was observed in EVs from cytokine-treated human islets. Nanoparticle tracking analysis showed no changes in EV quantity or size distribution in response to cytokine exposure, implicating transcript upregulation and changes in EV cargo as responsible for the observed increases. To assay changes in circulating EV miR-21, we performed longitudinal serum collections on NOD mice and insulitis resistant NOR controls, from 9 wks of age and until diabetes onset (defined as blood glucose > 200 mg/dL × 2, n = 7). Starting 3 weeks prior to diabetes

onset, EV miR-21 levels progressively increased in serum of diabetic NODs compared to age-matched NOR controls, peaking at a 10-fold increase from baseline levels. To validate relevance to human diabetes, serum EV miR-21 was assayed in samples collected from paediatric T1D patients at the time of diagnosis, as well as age-matched healthy controls (n = 19/group). Consistent with our NOD data, serum EV miR-21 was significantly increased in diabetic samples compared to controls. We propose that EV miR-21 may be a promising marker of insulitis and developing T1D in susceptible individuals. Ongoing studies will further define relationships between EV miR-21 content and β cell inflammation and death.

PT06.08

Circulating Tie2+ microvesicles as potential indicators of diabetic retinopathy progression

Aleksandra Tokarz[1], Anna ElżbietaDrożdż[2], Iwona Szuścik[3] and Ewa Stępień[2]

[1]Department of Clinical Biochemistry, Jagiellonian University Medical College, Krakow, Poland; [2]Department of Medical Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University, Krakow, Poland; [3]Private Ophthalmology Practice, OKO-LASER Outpatient Clinic

Introduction: Retinopathy is the most commonly occurring microvascular complication of diabetes, which is a major cause of vision loss. Although this complication covers substantially the retina, diabetic retinopathy (DR) has a systemic importance. The course of DR is very complex and multifaceted. In the initial phase of the dominant process is an increase in the permeability of the blood-retina barrier, inflammation and loss of endothelial function hypoxia. Angiopoietin-2 is considered to be involved in the increased endothelium permeability and dysfunction. Its autocrine action is regulated by angiopoietin receptor (Tie-2), present on the surface of endothelial cells and endothelial origin MVs.
Methods: DM patients (n = 61) aged 63 (59–68) and healthy controls (n = 25) aged 50 (45–56) were enrolled. The diagnosis and classification of retinopathy were carried out on the basis of the Polish Diabetes Association recommendations (2016). Finally, among examined DM patients, 7 had soft non-proliferative diabetic retinopathy (SNPDR), 5 had moderate non-proliferative (MNPDR), 13 had heavy non-proliferative (HNPDR) and 6 had PDR. MVs profiling ($Tie2^+$) in plasma was performed by means of Gigamix (BioCytex) calibrated CytoFLEX (Beckman Coulter). This study has permission of the Bioethical Committee of Jagiellonian University (KBET/206/B/2013).
Results: Ang-2 levels were significantly higher in DM patients when compared to healthy controls (3.5 [2.0–4.3] vs. 1.8 (1.5–2.1), p+ MVs than controls (p = 0.016); however, the number of platelet and endothelial origin MVs was the same. There was no correlation between Ang-2 and the total number of MVs and $Tie2^+$. The significant increase in the number of $Tie2^+$positive MVs was observed in HNPDR (293 [108–391] n/μL) to decrease in PDR (90 [70–198] n/μL).
Summary: The number of pro-angiogenic MVs is lower in DM patients then in control. The critical point in DR progression is the most advanced non-proliferative DR (HNPD), where the number of $Tie2^+$ MVs is significantly elevated.

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PT06.09

Urine extracellular vesicles transcriptome in diabetic kidney disease

Maija Puhka[1], Om Dwivedi[1], Carol Forsblom[2], Erkka Valo[2], Karina Barreiro[1], Harry Holthöfer[1], Per-Henrik Groop[2] and Leif Groop[1]

[1]Institute for Molecular Medicine Finland FIMM, University of Helsinki, Finland; [2]Folkhälsan Institute of Genetics, Folkhälsan Research Centre, Helsinki, Finland

Introduction: Diabetic kidney disease (DKD) lacks non-invasive early biomarkers.We examined the transcriptome of urine extracellular vesicles (EVs) via RNAseq as a biomarker for DKD. We also compared storage conditions of the urine samples (−20°C vs. −80°C) to clarify whether sample collections in −20°C can be used for biomarker discovery.
Methods: EVs were isolated from 24 h urine samples of 20 type 1 diabetic patients (T1D): 6 macro- and 2 microalbuminuric cases and 12 normoalbuminuric controls. EV quality was controlled with EM and Western blotting (WB). RNAs were profiled with Bioanalyzer Pico kit and subjected to RNAseq after cDNA library preparation with low amount protocols. Reads were aligned to human reference genome and counted with GENCODE gene annotations. Gene expression was measured as FKPM (Fragments per Kilobase of exon Per Million).
Results: EVs appeared typical at EM and positive for the EV-marker CD9 and kidney-derived podocalyxin in WB. RNA quantity and quality sufficed for RNAseq producing >15 million reads from all samples. Samples stored at different temperatures clustered in their own groups. On average, we detected expression (FKPM >1) of 13,161 genes. Analysis of 182 kidney-specific genes revealed that >70% (total 132) were expressed in EVs. Principal component analysis of these 132 genes discriminated macro- from normoalbuminuric T1D patients. Six genes were differentially expressed in DKD (p_{uncorr} < 0.001, fold change >1.5 or < 0.66) with the most striking difference in the KL gene previously linked to chronic kidney disease. Highly expressed genes in EVs (N = 5153, FKPM ≥ 10) were enriched in pathways of cellular metabolism, vesicle trafficking, mitochondrial and ribosome function.Pathway and gene enrichment analyses of all nominal differentially expressed genes implicated TGF-beta signalling, PI3K-Akt signalling and immune pathway in DKD (N = 956, p < 0.002)
Conclusion: Urine EVs can capture a significant part of the kidney-specific transcriptome and differentiate macro- from normoalbuminuric T1D patients. Technically, samples stored at different temperatures cannot be directly compared calling for meticulous standardisation of protocols. These should include comparison of, for example, EV isolation and storage methods to allow large-scale studies required for biomarker discovery.

LBP.16

Role of exosomal miRNAs in RPE cell mitochondrial dysfunction in AMD

Michael Paulaitis[1], Ju Young Ahn[1], Sayantan Datta[2], Elga Bandeira[3], Marisol Cano[2] and James Handa[2]

[1]Center for Nanomedicine at the Wilmer Eye Institute, Johns Hopkins University School of Medicine, MD, USA; [2]Wilmer Eye Institute, Johns Hopkins University School of Medicine, MD, USA; [3]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden

Introduction: Mitochondrial function declines with aging, and when significant, contributes to the onset of neurodegenerative diseases, such as Parkinson's and Alzheimer's disease, and age-related macular degeneration (AMD). Exosome formation/release is related to mitochondrial dysfunction through the lysosomal and exocytic pathways that process and eliminate intracellular fragments. Relevance to AMD is through retinal pigmented epithelial (RPE) cells, which maintain a healthy retina by phagocytosis of photoreceptor outer segments, an energy intensive process that requires highly functional mitochondria and a robust autophagic system for removing unwanted intracellular material. We hypothesize there cells with impaired mitochondria will release exosomes with a unique miRNA signature that reflects both mitochondrial breakdown within these cells and stress placed on the lysosomal and exocytic pathways, and as such, may be a diagnostic for AMD.
Methods: We screened for >700 human miRNAs in ARPE-19 cells, mitochondria isolated from these cells, and ARPE-19 exosomes characterized by their size distribution, morphology, and the presence of CD63. Validation of specific mitochondrial miRNAs (mito-miRs) and their presence in ARPE-19 exosomes was performed by qRT-PCR assay. ARPE-19 cells transfected with locked nucleic acid inhibitors targeted to specific mito-miRs served to validate their mitochondrial function. Mitochondrial injury was induced in the cells by treatment with rotenone, which impairs mitochondrial complex I.
Results: We identified *miR-494-3p* and *miR-579-3p* as mito-miRs that are also present at statistically significant levels in exosomes derived from untreated ARPE-19 cells. We also measured a significant enhancement in exosomal *miR-494-3p* in response to rotenone treatment of the ARPE-19 cells.
Summary/Conclusion: Our finding of enhanced levels of *miR-494-3p* in exosomes derived from rotenone-treated ARPE-19 cells identifies this mito-miR as a potential exosomal biomarker for AMD. The presence of this mito-miR in ARPE-19 exosomes also raises the possibility that

mitochondrial function in RPE cells can be regulated by exosome-mediated intercellular transfer of mito-miRs, such as *miR-494-3p*.

LBP.17

Salivary EV expression in traumatic brain injury

Mandy Pereira[1], Yan Cheng[1], Neha Raukar[2], John Reagan[3], Mark Dooner[4], W. Curt LaFrance[5], Matt Quesenberry[1] and Peter Quesenberry[6]

[1]Rhode Island Hospital/Alpert Medical School of Brown University, Department of Medicine, Divisions of Hematology/Oncology, RI, USA; [2]Rhode Island Hospital/Alpert Medical School of Brown University Emergency Medicine, RI, USA; [3]Rhode Island Hospital/Alpert Medical School of Brown University, RI, USA; [4]Brown University/Rhode Island Hospital Divisions of Hematology/Oncology, RI, USA; [5]Rhode Island Hospital/Alpert Medical School of Brown University, Psychiatry and Neurology, RI, USA; [6]Brown University/Rhode Island Hospital Department of Medicine, Divisions of Hematology/Oncology, RI, USA

Introduction: In 2013, 50,000 traumatic brain injury (TBI) related deaths occurred. Mild TBI (or concussions) is clinically difficult to diagnose due to limited sensitivity with CT and MRI. Studies have shown possible biomarkers in body fluids such as cerebral spinal fluid (CSF) and blood as predictive of degenerative brain disease in patients' post-traumatic brain injury (TBI). Increased levels of β-amyloid, and tau associated with Alzheimer's disease (AD) have also been seen in patients post-TBI (Blennhow 2010). For example, increased levels of Caspase-3, S100β, GFAP, and TrkB have been found in the brains of patients that died due to TBI (Staffa 2012) and found in blood and CSF samples. We wished to determine if aberrant levels of similar genes, or specific genetic profiles could be found in salivary extracellular vesicles (EVs) of subjects after TBI.
Methods: Saliva was collected from emergency room (ER) patients who either had a confirmed head impact or no recorded impact (as a control), and chronic concussion patients to isolate EVs. Healthy volunteers were used as a control. EVs were isolated via differential centrifugation and analyzed for mRNA and microRNA content using real time quantitative PCR.
Results: Concussion clinic patients had 14 microRNAs significantly changed. ER patients had significant elevation of 9 genes associated with AD, such as APLP2, MAPT, AND CSNK1D, and 12 inflammation genes such as ALOX5, ANXA3, CASP1. Concussion clinic patients had 21 AD genes elevated, such as APBA3, CAPNS2, CDK5R1 and 12 inflammation genes, such as ADRB1, ADRB2, and BDKRB1. The Wilcoxon sum test was used to compare gene expressions of patients to healthy controls.
Conclusion: Salivary EV profiling could be developed as a non-invasive test that might predict the development and progression of degenerative brain disease associated with TBI.
Funding: This project was supported by the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health through Grant Number (COBRE) P20GM103468 (PJQ) and the National Heart, Lungs, and Blood Institute (NHLBI) Grant T32HL116249.

LBP.18

ExRNAs in human cerebrospinal fluid are biomarkers for Alzheimer's disease

Julie Saugstad[1], Theresa Lusardi[2], Jay Phillips[3], Jack Wiedrick[3], Jodi Lapidus[3], Christina Harrington[3], Trevor McFarland[3], Babette Lind[3] and Joseph Quinn[4]

[1]Anesthesiology and Perioperative Medicine, Oregon Health and Science University, OR, USA; [2]Computational Biology, Oregon Health and Science University, OR, USA; [3]Oregon Health & Science University, OR, USA; [4]Neurology, OHSU School of Medicine

Introduction: Currently available biomarkers of Alzheimer's disease (AD) are limited. The discovery of extracellular microRNAs (miRNAs) in cerebrospinal fluid (CSF) raises the possibility that miRNA may serve as novel biomarkers of AD. We investigated miRNAs in CSF from living donors as biomarkers for AD.
Methods: We profiled miRNAs in CSF from 50 AD patients and 49 controls using TaqMan® arrays. Replicate studies on a subset of original CSF samples verified 20 high confidence miRNAs. Stringent data analysis using a four-step statistical selection process including log-rank and receiver operating characteristic (ROC) tests, followed by random forest tests, identified 16 additional AD miRNA candidates. Multi-marker modeling evaluated linear combinations of these miRNAs to ascertain classification performance, and this was compared to that of ApoE4 genotype. In addition, incremental improvement adding miRNA biomarkers to ApoE4 was assessed. Validation studies of 36 AD miRNA biomarker candidates on an independent set of 47 AD patients and 71 control CSF samples are complete, and classification performance of high-confidence miRNA biomarkers for AD ascertained using a targeted analytic pipeline to refine marker combination algorithms and suggest thresholds for positivity will be presented. The added value of ApoE4 genotype and other potential classifiers (i.e., Aβ:tau ratio) on biomarker performance will also be presented.
Results: We discovered 36 miRNAs that discriminate AD from control CSF. 20 of these retested in replicate studies verified differential expression between AD and controls. Stringent statistical analysis identified these 20 miRNAs, and 16 additional miRNAs, as candidate biomarkers for AD. Top-performing linear combinations of 3 and 4 miRNAs have AUC of 0.80–0.82. Addition of ApoE4 genotype to the model improved performance. Validation studies for the 36 AD miRNA biomarker candidates on a new and independent cohort to determine whether miRNAs in CSF, alone or in combination with other classifiers, can serve aa a biomarker for AD, will be presented.
Summary/Conclusion: CSF miRNAs can discriminate AD patients from controls. Combining miRNAs improves sensitivity and specificity of biomarker performance, and adding ApoE4 genotype, and possibly other classifiers, improves classification.
Funding: NIH NCATS UH2/3 TR000903 (JAS, JFQ)

Poster Session PT07 – EV Proteomics and Lipidomics
Chairs: Suresh Mathivanan and Alicia Llorente 5:15–6:30 p.m.

PT07.01

Lipidomic profiles of exosomes and microvesicles from human mesenchymal stem cell

Sicheng Wen[1], Patrycja Dubielecka-Szczerba[1], Michal Grzybek[2], Mark Dooner[1], Giovanni Camussi[3] and Peter Quensenberry[1]

[1]Brown University/Rhode Island Hospital, OR, USA; [2]Membrane Biochemistry, Paul Langerhans Institute Dresden, Medical Faculty TU Dresden, Dresden, Germany; [3]Department of Medical Sciences,University of Turin, Torino, Italy

Introduction: Extracellular vesicles (EVs), including exosomes and microvesicles (MVs), are the small spherical membrane particles released from cells, which have been shown to be involved in cell-to-cell communication and modification of the phenotype of target cells. Mesenchymal stem cells (MSCs) derived EVs have been shown to mediate reversal of different tissue injuries including kidney, brain, bone marrow and myocardium.
Methods: To advance our understanding of the biology and physiological functions of MSCs-EVs and to explore their therapeutic potential, we have used shotgun mass spectrometry to provide comprehensive characteristics of the lipid content of EVs isolated from MSCs. We designed our experimental strategy to answer two questions: (i) are MSCs-derived EVs differ from their parental cell line in terms of the lipid composition and (ii) are there differences in lipid compositions between various MSCs-derived EVs types.
Results: Three fractions of EVs including exosomes, MVs, and the combined fraction of exosomes and MVs were obtained from human MSCs using differential ultracentrifugation steps (2000*g*, 10,000*g* and then 100,000*g*). Lipidomic data reveal significant differences in lipid content within vesicle subtypes, and between EVs and parental MSCs. When compared with their parental cells, all three fractions of EVs are significantly enriched in lyso-phosphatidylcholine (LPC), sphingomyelin (SM), cholesterol esters (CE) and phosphatidylcholine-ether (PC(O-)), whereas levels of phosphatidylglycerol (PG), phosphatidylethanolamine-ether (PE(O-)) and triacylglycerol (TAG) are lower in all three fractions of EVs. In comparison to MSCs parental cells, no TAG and cardiolipin (CER) are present in exosomes, and only little amount of TAG present in MVs. Comparison of the lipidomic profiles of exosomes vs MV showed significant differences between these two fractions. Exosomes showed enrichment in LPC, LPC (-O) and SM, whereas MVs characterised enrichment in CER, PE(-O) and diacylglycerol (DAG). A principal component analysis clearly indicated different lipid profile between EVs and parent MSCs.
Conclusion: Our data indicate that both Exosome and MV membranes are enriched in certain classes of lipids, which may indicate potential role of these lipids in general function and biology of EVs.

PT07.02

Non-targeted metabolite profiling reveals differences in the lipid composition of extracellular vesicles derived from prostate cells grown in traditional 2D cultures versus in 3D bioreactor

Mari Palviainen[1], Jenna Pekkinen[2], Heikki Saari[3], Marjo Yliperttula[4], Kati Hanhineva[2], Maija Puhka[5] and Pia R-M. Siljander[6]

[1]EV-core, Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy and Institute of Molecular Medicine Finland FIMM, University of Helsinki, Finland; [2]LC-MS Metabolomics Centre, University of Eastern Finland, Finland; [3]Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki; [4]Division of Pharmaceutical Biosciences and Centre for Drug Research, Faculty of Pharmacy, University of Helsinki; [5]Institute for Molecular Medicine Finland FIMM, University of Helsinki, Finland; [6]Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

Background: Extracellular vesicles (EVs) have a lipid bilayer membrane that is structurally comparable to cell membranes making them optimal drug delivery vehicles. Since the production of EVs in traditional 2D cell culture setup results in relatively low amounts of EVs, 3D bioreactors have come more popular as a way to increase the EV yield. In addition to the EV yield and proteome, comparative analyses between 2D and 3D culture-derived EVs have been scarce. Since lipids play important roles in EV functions, we assessed how the cell culture setup affects the EV lipid metabolome.
Methods: Prostate cell lines PC-3, VCaP and PNT2 cells were grown in both traditional 2D cultures and in bioreactors. EVs were isolated from cell cultures using differential centrifugation at 20,000*g* (20 K pellet, microvesicles), and subsequently at 100,000*g* (100 K pellet, exosomes). EVs were characterised with nanoparticle tracking analysis (NTA) and western blotting, and the lipid metabolites were analysed using non-targeted LC-qTOF-MS approach.
Results: EV yield was superior from cells cultured in bioreactors compared to traditional 2D cultures. The size distribution of EVs did not differ between the 2D- and 3D-derived 20 K samples, but in the 100 K samples the EVs from all cell lines grown in the traditional 2D cultures were bigger that the EVs from bioreactors. More than 130 individual lipid metabolites were identified from all sample groups, belonging to glycerophospholipids, sphingolipids, sterol lipids and fatty amides. EVs derived from the cells grown in the traditional 2D cultures tended to have a broader spectrum of individual lipid metabolites than the EVs derived from cells grown in the bioreactors.
Conclusion: The results suggest that the environment where the cells are grown alters the EV features. Deeper metabolomics analyses will reveal information about the cell status and next we will study how these changes affect the functionality of EVs.

PT07.03

Quantitative comparison between small and large extracellular vesicles reveals enrichment of adhesion proteins in small extracellular vesicles

Lizandra Jimenez[1], Hui Yu[1], Andrew McKenzie[2], Qi Liu[1] and Alissa Weaver[1]

[1]Vanderbilt University, TN, USA; [2]Sarah Cannon Research Institute

Introduction: Extracellular vesicles (EVs) are important mediators of cell-cell communication due to their cargo content of proteins, lipids and RNAs. We previously reported smaller EVs, such as exosomes, promote a variety of aggressive cancer cell traits, such as cell motility and invasion. In contrast larger EVs, such as microvesicles, were not active in our systems. The goal of this study was to identify differences in the protein cargos of small and large EVs that may contribute to their different functional properties.
Methods: We utilised isobaric tag for relative and absolute quantitation (iTRAQ)-LC-MS/MS to perform a comprehensive comparison of protein cargos in small and large EVs obtained from the colorectal cancer line DKs-8. Statistically significant differences in proteins between the two EV types were identified by differential expression and gene set enrichment analysis methods. Proteins of interest were validated by Western blot analysis of EVs purified from the DKs-8 cells as well as from HT1080 fibrosarcoma cells.
Results: This proteomic analysis showed that small EVs were enriched in proteins associated with cell-cell junctions, cell-matrix adhesion and the exosome biogenesis machinery. In contrast, large EVs were enriched in proteins associated with ribosome and RNA biogenesis and processing, and metabolism. Western blot analysis confirmed the presence of integrins, thrombospondin and Ephrin receptors in small EVs. In addition, another highly abundant protein in the small EVs was arrestin-domain containing protein 1 (ARRDC1), which has been implicated in budding

of small microvesicles from the plasma membrane. We are currently trying to analyse the protein cargos carried by small EVs derived by ARRDC1-mediated budding versus exosomes.
Conclusion: iTRAQ is a useful method to identify protein differences in complex EV populations. Adhesion proteins appear to be particularly enriched in small EVs and may function to promote cell motility.

PT07.04

Advancement of multi-parametric profiling of extracellular vesicles: comparison of protein extraction by Laemmli and Trizol reagents using nanoLC-MS/MS

D. Craig Ayre[1], Andrew Joy[1], David Barnett[1], Anirban Ghosh[2], Rodney J Ouellette[2] and Stephen M. Lewis[2]

[1]Atlantic Cancer Research Institute, New Brunswick, Canada; [2]Department of Chemistry and Biochemistry, Université de Moncton, New Brunswick, Canada

Introduction: Extracellular vesicles (EVs) are anticipated to be valuable sources of disease-specific biomarkers, but yields are often limited by the amount of starting material. Trizol-based extraction can generate both RNA and protein fractions from the same EV sample, enabling a multi-parametric analysis that minimises time, cost, sample volume and variability. Here we compare the protein complement of EVs isolated from conditioned culture media of two breast cancer cell lines, MCF-7 and MDA-MB-231, using Laemmli and Trizol extraction.
Methods: EVs from two sets of biological triplicates (3 mL each of conditioned media) were isolated from each cell line using the Vn96 affinity peptide. For each set of triplicates, proteins were extracted from EV/Vn96 pellets using either Laemmli or Trizol buffer and subsequently digested for analysis by LC-MS/MS. Peptide extracts were separated by gradient nanoLC chromatography and detected using a hybrid quadrupole-Orbitrap mass spectrometer. LC-MS/MS data was searched against a Uniprot database using Proteome Discoverer 2.0. Aqueous phases of Trizol extracts were saved for RNA extraction.
Results:Trizol and Laemmli replicates were each analysed in technical triplicate by LC-MS/MS. Within a set of LC-MS/MS triplicates, approximately 75–81% of the identified proteins were found to be present in two out of three replicates. Comparisons of common protein lists between the two extraction methods show a lower degree of similarity than the technical replicates. In addition to comparing extraction methods, we also looked at similarities between cell lines in terms of gene ontology. No significant differences in cellular component ontology between the two extraction methods were observed for either of the cell lines.
Conclusion: Our results demonstrate that Trizol extraction of protein from EVs gives comparable results to Laemmli extraction while also allowing for parallel RNA isolation from the same sample. This method is particularly relevant to the development of multi-parametric protocols for liquid biopsy samples in clinical studies. A comparison of the RNA profile in relation to protein profile is in progress.

PT07.05

Comparative analysis of extracellular vesicle proteome and small RNA transcriptome reveals global abundance of RNA binding proteins but the relative depletion of miRNA related proteins and transcripts

Imre Mäger[1], Helena Sork[2], Yi Xin Fiona Lee[3], Henrik Johansson[4], Janne Lehtiö[4], Matthew J. Wood[T] and Samir EL-Andaloussi[1,2]

[1]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom; [2]Department of Laboratory Medicine, Karolinska Instiutet, Stockholm, Sweden; [3]Genome Institute of Singapore, Singapore; [4]Science for Life Laboratory, Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden

Introduction: Extracellular vesicles (EVs) are known to contain a range of bioactive molecules, including lipids, proteins and nucleic acid that could be transferred and result in an altered functional state of the recipient cell. Primarily due to that, there is an increasing interest in the detailed composition of these vesicular components, especially miRNAs, representing one of the most potent gene expression modulators. In addition, comparative "omics" analysis is encouraged to provide a global understanding of the mechanisms underlying vesicular cargo sorting.
Methods: Our study combines the proteome and small RNA transcriptome analysis of EVs extracted from conditioned medium of Hek293T and C2C12 cell lines using an ultracentrifugation protocol. Cell-line specificity and abundance profiling as well as gene ontology (GO) term annotation of the identified EV proteins were investigated and compared to the entries in the Vesiclepedia database. The aforementioned data was further matched with smallRNA sequencing results to understand the detailed composition of the vesicle associated small RNAs and match them to the abundance of the related proteins for integrated "omics" analysis.
Results: The study revealed a high abundance of proteins related to GO terms describing ribosomes and protein targeting to membrane. However, we observed a notable scarcity of proteins classified under GO terms describing miRNA related proteins. This corroborated with our transcriptomic data exposing an extreme abundance of ribosomal RNA fragments, however only under 1% of all RNA reads matched current miRNA annotations in miRBase.
Summary: This study outlines the combined proteomic and transcriptomic profile of EVs derived from two different cell sources, aiming to shed light to the interplay of vesicular components for future untangling of global molecular cargo sorting mechanisms.

PT07.06

Proteomic analysis of extracellular vesicles obtained from *Toxoplasma gondii* and toxoplasma-infected cells

Pryscilla F. Wowk[1], Maria Luisa Zardo[1], Halisson Tesseroli. Miot[1], Samuel Goldenberg[1], Paulo C. Carvalho[1] and Patricia A. Morking[1,2]

[1]Instituto Carlos Chagas/FIOCRUZ-PR, Curitiba, Brazil; [2]Institut Curie, Paris, France

Introduction: *Toxoplasma gondii* is an opportunistic pathogen, which is the causative agent of toxoplasmosis, a worldwide zoonosis. It is known that, like other protozoan, it produces functional extracellular vesicles (EVs). The effect of EVs derived from *T. gondii* on the immune response and its relevance in a physiological context is presently unknown. In order to bring new insights into the participation of EVs in the Toxoplasma dissemination and in the regulation of the host immune systems we disclose here the first proteomic profiling of *T. gondii* EVs (TgEV) compared to EVs isolated from a human foreskin fibroblast (HFF) infected (ICEV), and non-infected cells (NICEV) cultured in a vesicle-free medium.
Methods: *T. gondii* tachyzoites (RH strain) were maintained by serial passage in HFF monolayers at 37°C in a humid 5% CO_2 atmosphere. Three days after peal, a billion parasites were washed and cultured in DMEM HG for 2 h at 28°C. Supernatants free from parasites were filtered in a 0.45 µm membrane, and EVs isolation was performed using total exosome isolation kit. Dimensional characterisation was performed using NanoSight LM10. TEM was carried out in JEOL JEM-1400 Plus EM. Protein levels were assessed by the Qubit fluorometric quantification. Three EV sample preparations, independently obtained from *T. gondii*, HFF-infected, and non-infected cells were separated by SDS-PAGE. The gel lanes were excised, sliced and digested with trypsin. Five micrograms of protein were analysed in triplicate by LC-MS/MS in a Thermo Scientific Easy-nLC 1000 system coupled to a LTQ Orbitrap XL ETD. Peaklist picking, protein identification, were done using the MaxQuant version 1.5.0.25 and Pattern Lab platform.
Results: TEM shows vesicles of about 100 nm size for all samples. Proteomics analysis identified 346, 69 and 15 proteins as being unique to TgEV, ICEV and NICEV, respectively. When the common content from the three targets was analysed, a broad range of identified proteins correspond to classical EVs markers as annexins, HSP70, serpins, and tetraspanins. Data are available via ProteomeXchange (ID: PXD004895).
Conclusion: We present here the first characterisation of EVs protein contents and the contribution of the *T. gondii* and HFF cell in its formation. It is noteworthy that our proteomic data is in accordance to the legitimated proteins reported at EVpedia.

PT07.07

Proteomic analysis of extracellular vesicles derived from propionibacterium acnes

Jinseong Jeon, Jin Her and Changill Ban

Pohang University of Science and Technology, Pohang, Republic of Korea

Extracellular vesicle (EV) has been reported to conduct critical pathophysiological functions as an emerging mode of communication in bacteria. Recently, *Propionibacterium acnes*, an anaerobic Gram-positive human commensal found in the skin and gastrointestinal tract, has drawn increasing attention as an underestimated pathogen in a variety of diseases. For the comprehensive understanding of *P. acnes*, here we report the isolation of *P. acnes* EVs for the first time and identification of 252 vesicular proteins with high confidence using triplicate LC-MS/MS analyses. Comprehensive proteomic profiling reveals that *P. acnes* EVs harbour various proteins involved in biochemical processes, antibiotic resistance, bacterial competition, cell adherence, virulence and immunogenicity. We believe that this report will provide valuable information for investigating the biological role of *P. acnes* EVs and effective targets for developing clinical applications against *P. acnes*.

PT07.08

Proteomic analysis of mouse lung tissue-derived vesicles, a comparison of ultracentrifugation and density flotation isolation

Cecilia Lässer[1], Shintaro Suzuki[1], Kyong-Su Park[1], Ganesh Shelke[1], Lilit Hovhannisyan[2], Rossella Crescitelli[1] and Jan Lötvall[1]

[1]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden; [2]Institute of Molecular Biology, Armenian National Academy of Sciences, Yerevan, Armenia

Introduction: Analysis of the proteome of extracellular vesicles (EVs) is of great importance both to identify biomarkers of disease but also to understand cell-to-cell communication in diseased tissue. The aim of this study was to establish an isolation method that isolates lung vesicles of high purity for proteomic analysis.
Methods: A mouse model for allergic asthma was used by sensitisation and challenge of C57BL/6 mice to ovalbumin (OVA). Animals were sacrificed and lungs were removed and chopped in to smaller pieces that were incubated in medium for 30 minutes at 37°C and 5% CO_2. Vesicles were isolated from medium either by a differential ultracentrifugation protocol (UCF) or by an Optiprep density gradient protocol (OD). Isolated vesicles were evaluated by electron microscopy (EM) and the proteome was analysed with mass spectrometry (LC-MS/MS).
Results: EM showed that both protocols isolated vesicles that where on average 40–200 nm in size. LC-MS/MS identified 1223 and 1383 proteins in the UCF and OD vesicles, respectively. Out of these, 989 proteins were detected in both samples and 88 of the top 100 exosomal proteins from the database EVpedia was identified here. Using GO Term finder it was shown that the 989 common proteins were most significantly associated with the cellular component, "extracellular exosome", "focal adhesion" and "membrane". The 398 uniquely identified proteins in the OD vesicles were associated with "extracellular exosome" and "membrane", while the 234 uniquely identified proteins in the UCF vesicles were associated with "proteasome complex" and "cytoplasm".
Conclusion: This study shows that EVs can be isolated directly from lung tissue, and these vesicles contain previously identified EV proteins. Both protocols can be used for the isolation of tissue-derived vesicles. However, flotation removes a number of contaminant proteins, including those related to the proteasome and furthermore it enriches for protein associated with membrane.

PT07.09

Proteomic analysis of exosomes derived from acute myeloid leukaemia as maturation

Jihye Hong[1] and Kwang Pyo Kim[2]

[1]KHU, Seoul, Republic of Korea; [2]Department of Applied Chemistry, College of Applied Sciences, Kyung Hee University, Seoul, Republic of Korea

Introduction: Acute myeloid leukaemia (AML) is a malignant disease categorised by blocking monocyte differentiation and maturation as hematopoietic cells. AML is divided into several subtypes by degree of differentiation. Only a few proteomic studies of subtype-specific AML have been studied, and proteomic studies AML exosome are still insufficient. As exosomes reflect the nature of the original cell and convey cellular information, it is important to profile and compare exosomal proteome changes to understand pathophysiology of AML differentiation.
Methods: To elucidate the proteomic characteristics of the exosome from AML, we isolated exosomes using size-exclusion chromatography (SEC) from three subtypes of human AML according to FAB classification, acute promyelocytic leukaemia (HL60, M3), acute myelomonocytic leukaemia (KG-1, M4), acute monocytic leukaemia (THP-1, M5). For quantitative comparison, we analysed the protein profiles using the isobaric tag based tandem mass tag (TMT) labelling and liquid chromatography-tandem mass spectrometry (LC-MS/MS).
Results: A total of 2341 proteins were identified in all three groups. The commonly identified proteins were enriched in the categories of extracellular exosome and membrane and engaged in the pathways of focal adhesion and ECM-receptor interaction. And the protein profiles of each group were compared. The 496 proteins of M3 and M4, 325 proteins of M3 and M5 and 560 proteins of M4 and M5 were differentially expressed with a 1.5-fold change ($p < 0.05$). Gene ontology analysis of DEP found characteristic changes for each AML including cell and cell adhesion and SRP-dependent cotranslational protein targeting to membrane between M3 and M4, response to estradiol and lectin pathway between M3 and M5, and protein folding and retrograde vesicle-mediated transport for M4 and M5.
Conclusion: In the present study we performed proteome profiling of exosomes isolated from different AML cell lines. Also we compared enriched proteins in each AML cell lines in different maturation stages. Understanding maturation specific biological processes in AML cell lines could provide pathophysiological regulating factors for AML maturation.

PT07.10

The impact of oncogenic EGFRvIII on the proteome of extracellular vesicles released from glioblastoma cells

Dong-Sic Choi, Laura Montermini and Janusz Rak

The Research Institute of the McGill University Health Centre, Quebec, Canada

Glioblastoma multiforme (GBM) is the most common, highly invasive, and aggressive astrocytic brain tumour associated with poor prognosis. EGFR is amplified in a subset of GBMs and influences the invasion and proliferation of tumour cells. EGFR amplification is also often accompanied by gene rearrangements leading to the expression of constitutively active oncogenic mutant, EGFR variant III (EGFRvIII). In addition to intrinsic transformation of GBM cells themselves, EGFRvIII may also act in a non-cell-autonomous manner by virtue of intercellular trafficking of this receptor between cellular populations as cargo of extracellular vesicles (EVs). Notably, EGFRvIII may also influence EV biogenesis and alters the expression of multiple genes, but links between these events are poorly understood. To better understand how EGFRvIII contributes to tumour aggressiveness mediated by EVs, we investigated the effect of this oncogene on the EV protein composition. Thus, we employed the quantitative proteomics to analyse EVs derived from indolent parental U373 glioma cells and their EGFRvIII-expressing isogenic counterparts (U373vIII). EVs were purified using Optiprep density gradient ultracentrifugation and analysed with an UHPLC-Orbitrap Fusion Tribrid mass spectrometer. Compilation of three experimental replicates revealed remarkable changes in the expression profiles of the EV proteins, as well as changes in the release rate and concentrations of secreted EVs. For example, U373vIII-derived EVs exhibited a distinct profile of integrin expression, including elevated content of integrin α6β4, known to direct EVs to the lung. In contrast, parental U373 derived EVs carried integrin αVβ5, known to direct EVs to the liver. Thus, while GBMs generally do not metastasise to these respective organs their EVs may

home to these sites and contribute, in an oncogene-specific manner, to systemic pathologies associated with brain tumours (inflammation, thrombosis). Moreover, U373vIII cells secreted EVs contained high levels of other invasion-promoting proteins including CD44, CD151, BSG. In conclusion, our results suggest that oncogenic EGFRvIII profoundly impacts the proteome of EVs released by GBM cells, and may define their biological activities beyond the content of EGFRvIII oncoprotein itself.

PT07.11

Diabetic microenvironment alters circulating microparticle protein composition

Maddison Turner[1], Jean-Francois Thibodeau[1], Chet Holterman[1], Christopher Kennedy[2] and Dylan Burger[2]

[1]University of Ottawa, Canada; [2]Kidney Research Centre, Ottawa Hospital Research Institute, University of Ottawa, Canada

Background: People with diabetes are three times more likely to develop cardiovascular complication, however the molecular alterations responsible for this increased cardiovascular risk are not fully understood. As microparticle (MP) composition may reflect underlying pathology we examined the protein composition of circulating MPs isolated from OVE26 type 1 diabetic mice their wildtype (WT), non-diabetic litermates.
Methods: Platelet-free plasma samples were obtained from male OVE26 type 1 diabetic WT non-diabetic mice (20 weeks) by cardioac puncture following baseline characterisation. Circulating MPs ewre isolated by differential centrifucation and protein composition was assessed via mass spectrometry (MS). Gene Ontology pathway analysis was used to identiy signalling pathways with associated with the dysregulated proteins (>2 proteins/pathway).
Results: Compared with their age-matched WT littermates, OVE26 mice (20 weeks) displayed increased plasma glucose levels (29.9 ± 0.8 vs 11.3 ± 0.7 mM, $p < 0.001$, $n = 3$), decreased body weight (27.3 ± 0.9 vs. 32.4 ± 1.2 g, $p < 0.05$) and ~3-fold increase in urinary albumin/creatinine ratio ($p < 0.01$). Blood pressures were not significantly different between OVE26 and WT mice (123.5 ± 8.8 vs 115.6 ± 5.7, $p = 0.51$). MS of plasma-derived MPs identified 396 independent proteins with at least two peptides per protein with an average sequence coverage of 16%. 87 proteins were significantly downregulated while 21 were significantly upregulated in the diabetic mice using a 1.5-fold cut-off and $p < 0.05$ (Fisher's exact test). Functional pathway analysis revealed that MPs obtained from diabetic mice were enriched in proteins associated with blood coagulation, glucose metabolism, apoptosis and inflammation.
Conclusions: Taken together our results suggest that MPs obtained from diabetic mice display a distinct protein composition compared with MPs isolated from non-diabetic mice. In particular, diabetes-induced MPs are enriched in proteins associated with thrombosis, apoptosis, and inflammation. Further assessment of specific proteins involved in these processes may provide novel insights into the pathogenesis of vascular injury in diabetes.

PT07.12

Compositional changes in ageing platelet concentrate

Sami Valkonen[1,2], Feven Tigistu-Sahle[3], Birte Mallas[2], Minna Holopainen[2], Anne Valkeajärvi[2], Kaija Javela[2], Reijo Käkelä[2], Pia R-M. Siljander[1] and Saara Laitinen[2]

[1]Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland; [2]Finnish Red Cross Blood Service, Helsinki, Finland; [3]Department of Biosciences, University of Helsinki, Helsinki, Finland

Introduction: Adverse transfusion reactions are a clinically relevant problem of platelet concentrates and a link with the storage time of the concentrate and an increased incidence of adverse transfusion reactions has been proposed. Although measurements to monitor platelet activation have been developed, no clear indicators for platelet activation in the concentrates or good quality controls exist. If the compositional changes of ageing platelet concentrate were understood better, monitoring could be improved and the storage time of platelet concentrates could be extended from the current standard, 5 days.
Methods: Alterations in the composition of platelet concentrate were studied by determining (1) platelet activation state by CD62P-exposure and soluble glycoprotein V, (2) the concentration and size distribution of platelet-derived extracellular vesicles (EVs) and (3) changes in glycerophospholipid content and signalling lipid species of whole product, platelets and EVs. In total, 10 platelet concentrates were studied on days 1, 2, 5 and 8.
Results: From days 1–8, platelet activation increased according to both indicators. The EV in the concentrates increased on average 800% and the size of the produced EVs increased as a function of time. The whole concentrate, platelets, and EV resembled each other well in the main class level of lipids and only minor changes occurred in them as a function of time. However, when the components were compared in lipid species level, some differences were observed. Also, a time-dependent increase in both pro-resolvin and pro-inflammatory lipid mediators was observed, although only a few signalling lipid species were present in detectable amounts.
Conclusion: Compositional changes in lipids do occur in ageing platelet concentrates and further studies are needed to link these changes with the possible functional effects, as they may be relevant for the adverse or beneficial effects of platelet transfusions.

LBP.19

Acoustic Electrospray Mass Spectrometry of Extracellular Vesicle Lipids

Xabier Osteikoetxea[1], Martin Bachman[2], Ian Sinclair[3], Nikki Heath[1], Lois Grant[2], Niek Dekker[1], Ross Overman[2] and Lorenz Mayr[2]

[1]Astrazeneca; [2]AstraZeneca; [3]Associate Principal Scientist

Introduction: In the last decade the field of extracellular vesicle research has attracted much interest due to several studies showing that these particles carry and protect from degradation various proteins and RNAs and thus participate in diverse biological processes. Recent studies also point towards the potential of utilizing extracellular vesicles for diagnostics and therapy. In contrast to the wide availability of proteomic and transcriptomic data there are still relatively few reports on the lipid content and composition of extracellular vesicles. Here we aim to adapt a high throughput mass spectrometry approach to investigate the lipid composition of extracellular vesicles secreted by various cell lines.
Methods: Extracellular vesicle subpopulations (apoptotic bodies, microvesicles and exosomes) were isolated from cell line conditioned media by differential centrifugation and filtration. Extracellular vesicles were also characterized by nanoparticle tracking analysis and the presence of vesicle markers was assessed by western blot. Finally, extracellular vesicles were subjected to high throughput acoustic electrospray mass spectrometry for analysis of lipid composition.
Results: We found that acoustic electrospray was suitable for transfer of extracellular vesicles to the mass spectrometer allowing for fast label free analysis of lipids from small sample volumes. We observed hundreds of features in both positive and negative ion modes in the mass range of 400 to 1000 Da, mainly belonging to glycerophosphocholines, sphingomyelins and other glycerophosphate derivatives. The MS signal was significantly enhanced by adding ether to the well and spraying directly from the liquid-liquid interface. We found that extracellular vesicle subpopulations and their releasing cells differ in their lipid composition and also in the ratio of sodium and potassium adducts they form for identical species. Principle-component analysis was used to analyze and visualize spectral differences.
Summary/Conclusion: We have established acoustic electrospray mass spectrometry as a suitable high-throughput strategy for extracellular vesicle lipid analysis and found that cells and distinct extracellular vesicles subpopulations differ in their lipid composition.

Poster Session PT08 – EVs in Viral and Bacterial Infections
Chairs: Cherie Blenkiron and Metka Lenassi 5:15–6:30 p.m.

PT08.01

Role of circulating Epstein–Barr virus-encoded microRNAs in immune evasion

Manuel Albanese, Kathrin Gärtner, Corinna Hüls and Wolfgang Hammerschmidt

Department of Gene Vectors, Helmholtz Zentrum München, München, Germany

Epstein–Barr virus (EBV) is a prevalent herpesvirus and infects the majority of the human population. EBV causes a latent infection in its host for a lifetime, which is generally asymptomatic and governed by an efficient T cell control. In contrast to other herpesviruses, EBV encodes only three proteins, which act as immunoevasins. Among these genes, two viral immunoevasins, BNLF2a and viral IL-10, inhibit the recognition of infected cells by EBV-specific effector T cells and natural killer cells, respectively, but these two viral proteins are insufficient to prevent T cell recognition.
Twnety-five miRNA precursors have been identified in EBV, which are reported to interfere with cell death, innate immune responses and inflammation. We recently demonstrated that EBV miRNAs inhibit antiviral T cell responses early in infection acting as important immunoevasins. They efficiently inhibit the antigen presentation of EBV-infected B cells to CD8+ and CD4+ T lymphocytesthrough multiple mechanisms contributing to the maintenance of a lifelong infection.
It is known that EBV's miRNAs are also released from EBV infected B cells via extracellular vesicles (EVs) and taken up by surrounded antigen presenting cells.
In this study, we investigate if these viral circulating miRNAs can be transfered from cell to cell and if they are able to act as immunoevasins also in recipient cells.
EVs secreted by B cells infected with an EBV lab strain or with a mutant virus deficient of all miRNAs are isolated using a combination of differential centrifugation, ultracentrifugation, ultrafiltration and density gradient, and characterised by nanoparticle tracking analysis (NTA), AMNIS Imagestream, western blotting and quantitative PCR.
We found that that EVs secreted by infected B cells contain mature EBV's miRNAs which are taken up by several cell types but to different extents. Our data suggests that viral miRNAs released by infected B cells influence the environment and can support the virus to evade elimination in the host in spite of strong adaptive cellular immune responses. Further investigations are required to completely unravel the impact of EBV microRNAs in the different recipient cells and whether they act through the same mechanisms as in infected B cells.

PT08.02

Recombinant extracellular vesicles (EVs) are a tool to study the function of packaged viral RNAs from Epstein–Barr virus (EBV)

Corinna Hüls and Reinhard Zeidler

Department of Gene Vectors, Helmholtz Zentrum München, München, Germany

Introduction: Extracellular vesicles (EVs) are able to transfer proteins, lipids and nucleic acids. Moreover, they share similarities with enveloped viruses and virus-like particles. The human herpesvirus Epstein–Barr virus (EBV) packages its DNA genome as well as viral RNAs into virions and transfers them to their target cells where they are instantly functional. Until today, the biological function of packaged viral RNAs remains unclear. By using recombinant EVs, harbouring selected viral RNAs we investigated the role of transduced viral RNAs (tvRNAs) for the early phase of EBV-infection. We focused on the viral transactivators BZLF1 (Z) and BRLF1 (R) that are important for the initiation of a pre-latent phase and trigger the viral transcription during the early phase of infection.
Methods: Recombinant EVs were generated by transfecting HEK293 producer cells with plasmids encoding for Z and R and an additional plasmid encoding for the viral glycoprotein 350 (gp350) that mediates the B cell tropism to engineered EVs. EVs were purified via serial centrifugation, including ultracentrifugation, and filtration. Characterisation was performed by dot blot, flow cytometry and RNA isolation, followed by qRT-PCR. To investigate the impact of the tvRNAs on infected cells, primary human B cells were incubated with EVs and analysed by RNA sequencing.
Results: The recombinant EVs were positive for several EV-associated proteins (CD63, CD81) and the viral gp350 in dot blot and flow cytometry. The concentration was determined by nanoparticle tracking analysis and packaging of viral RNAs was successfully proven by qRT-PCR. Preliminary experiments showed effects on the expression of downstream genes after incubation with EVs carrying both viral transactivators. RNA-sequencing will reveal the influence on the cellular expression pattern.
Conclusion: We could show that recombinant extracellular vesicles provide the possibility to study individual sets of particles containing viral RNA and their impact on the early phase of infection and the establishment of latency of EBV.

PT08.03

Nef-exosomes as putative biomarkers for inadequate treatment regimen of HIV-1 infected individuals

Jana Ferdin[1], Katja Goričar[1], Vita Dolžan[1], Ana Plemenitaš[1], Jeffrey N. Martin[2], B. Matija Peterlin[3], Steven G. Deeks[3] and Metka Lenassi[1]

[1]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia; [2]Department of Epidemiology & Biostatistics, University of California San Francisco, USA; [3]Department of Medicine, University of California San Francisco, USA

Introduction: Antiretroviral therapy (ART) reduces plasma HIV-1 RNA levels to below the limit of detection in most infected individuals, however virus persists in cellular reservoirs and contributes to diverse non-AIDS-related disorders (cancer, cardiovascular disease, neurocognitive disorders). Viral proteins, like Nef, can promote this through chronic inflammation and/or with direct toxic effects. Nef protein is a key HIV-1 pathogenic factor, as it affects cellular signalling and metabolism, and viral infectivity. It is released from HIV-1 infected primary T cells with exosomes, which were also detected in plasma of HIV-1 infected individuals. The aim of this study was to explore if Nef-exosomes are present in plasma of aviremic HIV-1 infected individuals and if their levels correlate with patient's clinical status.
Methods: Plasma Nef-exosome levels were measured indirectly with Nef-ELISA assay. In total 134 subjects from the SCOPE cohort were included: 26 uninfected, 28 viremic (non-controllers) and 80 aviremic (38 treated (ART), 42 elite controllers (EC)). All subjects were characterised with respect to age, gender, ethnicity, HIV status (CD4+ and CD8+ counts, and HIV-1 RNA level) and ART regimen. Statistical analysis was performed using IBM SPSS Statistics (v19.0). All subjects provided informed consent and the parent study was approved by the UCSF Committee on Human Research.
Results: Protein Nef was detected in 63 subjects: 23 non-controllers (Mdn = 11.6 ng/ml), 18 ART (Mdn = 8.3 ng/ml) and 22 EC (Mdn = 8.8 ng/ml). Nef concentration positively correlated with HIV-1 RNA level (p = 0.048) only in non-controllers, which implies that the source of plasma Nef in aviremic individuals are not viruses. In ART treated subjects, plasma Nef correlated positively with protease inhibitors (PI)-based ART (OR (95% CI) 4.28, p = 0.045), whereas Nef negatively correlated with non-nucleoside reverse transcriptase inhibitors (NNRTI)-based ART (OR (95% CI) 0.11, p = 0.005).
Conclusion: Nef is present in plasma of half of aviremic HIV-1 infected individuals and is, based on literature, most likely part of exosomes. In ART-treated patients, plasma Nef correlates with the drug regimen.

Therefore, Nef-exosomes could be used as biomarkers to predict inadequate treatment with ART to prevent later health complications.

PT08.04

Single-particle characterisation: discriminating enveloped viruses and extracellular vesicles by flow virometry

Tyler M. Renner, Vera A. Tang and Marc-Andre Langlois

University of Ottawa, Canada

Introduction: Discriminating between host-derived extracellular vesicles (EVs) and enveloped viruses, such as retroviruses, represents a major technical challenge. In addition to being similar in size and density, these particles often have overlapping biogenesis and release pathways, and thus also share similar surface markers. As such, they often co-purify even under the most stringent methods, thereby compromising the results of single-particle characterisation studies.
Technological advancements are pushing single-particle characterisation techniques to the nanometre level. However, the challenge of discriminating between retroviruses and EVs remain. Surface antigen staining approaches with labelled antibodies have yet to provide a suitable solution, as antibodies themselves are nanometre-sized and have a propensity to aggregate the particles they target. Here we set out to develop new flow cytometry-based approaches to distinguish retroviruses from EVs and characterise the different EV sub-populations released during infection.
Methods: NIH 3T3 cells were infected with Moloney murine leukaemia virus (MLV) with an eGFP insertion in its surface envelope glycoprotein. Infected cells were stained with lipophilic tracer dye DiD. EVs and MLVs produced were analysed directly from the cell supernatant by nanoscale flow cytometry, also known as flow virometry, on a BD SORP LSRFortessa (300 mW 488 nm laser). Laser power and detector voltages were optimised for resolution of particles of interest from noise. A newly developed biological ladder enabled a more accurate evaluation of the sizes of particles being analysed by side scattered light
Results: Our approach detects the release of DiD+ EVs from uninfected cells. Infected cells produced large amounts of eGFP+ particles which were associated with a two fold increase in eGFP-DiD+ EVs. Interestingly, the presence of eGFP-DiD+ particles suggests that some populations of EVs did not bud at the cell surface or acquire the viral envelope glycoprotein.
Conclusion: We have developed methods to indirectly label single-particle EVs and viruses without the use of antibodies. These approaches allow us to characterise and quantify populations of submicron-sized particles released in the context of a viral infection.

PT08.05

Characterisation of extracellular vesicles purified from HIV-1 Nef overexpressing HEK293 cell supernatants

Julia L. Sanwald[1], Alexandra Boeske[2], Andreas Weber[3], Payam Akhyari[3], Silke Hoffmann[2] and Dieter Willbold[1]

[1]Institut für Physikalische Biologie, Heinrich Heine University Düsseldorf, Düsseldorf, Germany; [2]Institute of Complex Systems (ICS-6), Research Centre Jülich, Jülich, Germany; [3]Department of Cardiovascular Surgery, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Human immunodeficiency virus type 1 (HIV-1) accessory protein Nef (negative factor) provokes many pathogenic effects during acquired immunodeficiency syndrome progression. Amongst others, Nef, which has no signal peptide sequence, induces extensive secretion activities including its own unconventional protein secretion. Distribution of Nef via extracellular vesicles (EVs) is regarded as a crucial pathogenesis-relevant function. To date knowledge about the respective secretion path(s) is insufficient.
Our data demonstrate that Nef secretion strictly depends on the availability of at least one of the three human GABARAPs, a protein family involved in intracellular transport of vesicles and autophagosome formation. All GABARAPs exhibit direct Nef interaction, for which tryptophan 13 of Nef is essential. Here, we characterise EV pools obtained from untransfected HEK293 and cells overexpressing Nef wild type (WT), the GABARAPs-binding deficient variant Nef(W13A) or another secretion relevant variant, Nef(SMR5A). EVs were isolated with precipitation and ultracentrifugation based methods. Vesicle count and size distribution were determined by nanoparticle tracking analysis (NTA).
In accordance with Nef's function as secretion inducer, an increase in vesicle count was obtained for cells expressing Nef(WT) but also its variants. Applying density gradient centrifugation in combination with immunoblotting against Nef and EV markers the density distribution of the EVs was analysed and compared. In parallel, we performed time-resolved imaging of FM1-43 stained cells overexpressing Nef(WT) or Nef(W13A). In Nef(WT) expressing cells we found extensive, Nef containing bleb-like membrane patches. Nef(W13A) expressing cells produced smaller vesicles that possibly passed the plasma membrane in a more scattered manner.
This hints towards the existence of more than one secretion pathway used by Nef. Since we found the GABARAP-binding deficient Nef (W13A) in EVs, too, obviously not each Nef secretion path depends on the observed direct Nef-GABARAP interaction. Identification or separation of EV subpopulations specific for the different Nef variant expressing cells by size or density was not feasible, what can have several reasons. Proxitome based techniques may help to overcome this issue.

PT08.06

Ceramide- and CD63-dependent trafficking of Epstein–Barr virus LMP1 to extracellular vesicles

Sara B. York, Stephanie N. Hurwitz, Dingani Nkosi, Xia Liu and David G. Meckes

Florida State University College of Medicine, FL, USA

Introduction: Epstein–Barr virus (EBV) is a human herpesvirus that is associated with a multitude of epithelial and lymphoid cancers. Latent membrane protein 1 (LMP1) encoded by EBV is expressed in most EBV-associated cancers and is believed to be the major viral oncogene. LMP1 is secreted from infected cancer cells in small membrane-enclosed extracellular vesicles (EVs). LMP1-modified EVs can inhibit immune cell function and enhance cell growth and migration. In spite of the potential significance of extracellular LMP1, very little is known about how this viral protein traffics to vesicles, especially within lymphoblastoid cells. Recently, the tetraspanin protein CD63 has been found to form a complex with LMP1 and knock-out of CD63 in epithelial cell lines resulted in a reduction of exosomal LMP1. In certain cell lines, CD63 is trafficked to EVs through a ceramide-dependent mechanism. Therefore, we hypothesise that interaction with CD63 in ceramide-rich microdomains drives the trafficking and incorporation of LMP1 into EVs.
Methods: EVs from an EBV-infected lymphoblastoid cell line (LCL) were purified on density gradients to examine vesicle subpopulations containing LMP1. To analyse the effects of CD63 on exosome secretion and protein trafficking, CRISPR/Cas9 technology was used to knockout CD63 in LCLs. The requirement for ceramide in LMP1 exosomal trafficking was tested using GW4869.
Results: LMP1 was determined to be secreted by LCLs in small CD63-enriched exosome populations by gradient purification. Nanoparticle tracking analysis of EVs from CD63 CRISPR cells demonstrated a significant decrease in relative particle secretion. Similarly, decreases in vesicle secretion were found following GW4869 treatment. Immunoblotting of EV lysates revealed a reduction in exosomal LMP1 from both CD63 CRISPR and GW4869-treated cells.
Conclusion: Altogether, these data reveal that efficient secretion of LMP1 into small EVs from infected cells requires CD63 and ceramide.

PT08.07

Epstein–Barr virus LMP1 extracellular vesicle sorting is mediated by the N-terminus and transmembrane domains

Dingani Nkosi, Lauren A. Howell, Mujeeb Cheerathodi, Stephanie N. Hurwitz, Deanna C. Tremblay, Xia Liu and David G. Meckes

Florida State University College of Medicine, FL, USA

Introduction: The Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1) is released from latently infected tumour cells in small

membrane-enclosed vesicles called exosomes. Accumulating evidence suggests that LMP1 is a major driver of exosome content and functions. LMP1-modified exosomes have been shown to influence recipient cell growth, migration, and differentiation, in addition to regulating immune cell function. Even though the importance of LMP1-modified extracellular vesicles (EVs) on the infected microenvironment is well recognised, very little is known about how this viral protein enters or manipulates the host exosome pathway.
Methods: In this study, LMP1 deletion mutants were generated to assess protein regions required for EV trafficking. Following transfection of LMP1 plasmids, cell-derived extracellular vesicles were collected by differential centrifugation and levels of specific cargo were evaluated by immunoblot analysis.
Results: The results demonstrate that together the N-terminus and specific domains within the transmembrane regions of LMP1 are required for efficient sorting into the exosome pathway. Consistent with these findings, a mutant lacking the N-terminus and transmembrane domains 1 through 4 (TM5-6) that fails to be packaged into EVs exhibited higher co-localisation with endoplasmic reticulum and early endosome markers when compared to the wild-type protein. Other mutations within LMP1 resulted in enhanced levels of secretion, alluding to potential positive and negative regulatory mechanisms for LMP1 extracellular vesicle sorting. Surprisingly, TM5-6 maintained the ability to co-localise and form a complex with the tetraspanin CD63, an abundant exosome protein that is important for the incorporation of LMP1 into exosomes.
Conclusion: These data suggest new functions of the N-terminus and transmembrane domains in the role of LMP1 intra- and extracellular trafficking that are likely downstream of an interaction with CD63.

PT08.08

Proteomic analysis of the CD63 interaction network reveals important functions of CD63 in LMP1-dependent protein trafficking

Mujeeb Cheerathodi, Xia Liu and David G. Meckes

Florida State University College of Medicine, FL, USA

Introduction: CD63 is a common exosome marker belonging to the tetraspanin family of proteins, which are important in extracellular vesicle cargo sorting and protein trafficking within the cell. Indeed, our previous work has demonstrated the importance of CD63 in exosomal targeting and subcellular localisation of the Epstein–Barr virus oncoprotein LMP1, and in positively regulating small extracellular vesicle production. However, very little is known about the protein-protein interactions that could be driving these important CD63 functions. Here we sought to utilise the recently developed proximity-based BioID approach to identify CD63 interacting proteins and to further evaluate how this interactome changes in the presence of LMP1.
Methods: CD63 interacting proteins were identified using BioID pull down with streptavidin beads followed by LC-MS/MS and further analysed using DAVID and FunRich bioinformatics programs. Extracellular vesicle content was analysed by immunoblot following ExtraPEG and ultracentrifugation enrichments.
Results: Mass spectrometry analysis detected more than one thousand potential direct or indirect CD63 interacting partners. Bioinformatics analysis revealed the identified proteins are enriched in protein trafficking, vesicle transport, exosome targeting and cell signalling. Selected known and novel interaction partners were verified by immunoblot analysis. Interestingly, two proteins previously known to be regulated by LMP1, EGFR and vimentin, were identified as CD63 interacting proteins and efficient trafficking of these proteins to extracellular vesicles required CD63.
Conclusion: Overall, this study defines the protein interaction network of CD63 and provides new insights into the functions of CD63 in protein trafficking, vesicle biogenesis, and signal transduction in context of LMP1 expression. Based on these findings, it is likely that CD63 is an important host factor in LMP1-driven modification of extracellular vesicle content and function.

PT08.09

The inflammatory and immunological roles of *S. aureus* derived exosome-like vesicles in septic arthritis

Farah Fatima[1], Majd Mohammad[1], Abukar Ali[1], Muhammad Nawaz[1,2], Hadi Valadi[1], Manli Na[1] and Tao Jin[1]

[1]University of Gothenburg, Gothenburg, Sweden; [2]University of Sao Paulo, Sao Paulo, Brazil

Introduction: *Staphylococcus aureus* is the most common pathogen of septic arthritis worldwide with increasing incidence each year. Virulence factors from *S. aureus* trigger host immune response and propagate infection severity. It has been shown that *S. aureus* secrete exosome-like extracellular vesicles (EVs) that not only mediate host-pathogen interaction but also serve as modulators of infection. However, their role in *S. aureus* induced septic arthritis has not been studied so far. In this study we explored the role of *S. aureus* derived EVs for stimulating immune responses and infection in a mice model of septic arthritis.
Methods: *S. aureus* strain Newman was cultured overnight and EVs were isolated by ultracentrifugation and filtration. Mice splenocytes were cultured *in vitro* and were stimulated with various doses of EVs. Cell proliferation was observed and cytokines level was measurement by ELISA. EVs were injected intra-articularly to induce local joint inflammation. Histopathological analysis of knee joints was performed to evaluate synovitis and joint erosion.
Results: EVs induced a differential production of cytokines as compared to controls with significantly elevated levels of TNF-α and IL-6 in a dose dependent manner. Histopathological analysis of intra-articularly injected knee joints showed degree of synovitis.
Conclusion: *S. aureus* derived EVs could potentially provoke inflammatory and immunological responses both *in vitro* and *in vivo*. Collectively, our results suggest that *S. aureus* secreted EVs are functional extensions of *S. aureus* acting as virulence factors however to understand the underlying mechanisms further studies are needed.

PT08.10

Differential diagnosis of pulmonary tuberculosis and lung cancer by microRNAs in serum extracellular vesicles

Taixue An, Sihua Qin, Yong Xu, Yiyao Huang, Shaopeng Li and Lei Zheng

Department of Laboratory Medicine, Southern Medical University Affiliated Nanfang Hospital, Guangdong, China

Introduction: As the sensitivity of imagology enhanced the detection rate of pulmonary nodules increases rapidly. Differentiating lung cancer and tuberculosis (TB) have become a troubling problem. It has been universally reported that miRNAs in extracellular vesicles (EVs) are aberrant in pathologic state. It is, therefore, meaningful to explore if miRNAs in EVs derived from serum can serve as a biomarker to differentiate these two diseases.
Methods: We collected the serum of 204 patients with lung cancer, and 132 patients with TB and 181 health people. Classic three-step strategy was employed to find the differential diagnostic biomarker. Serum EVs were isolated with regents and characterised by WB, nanosight, electron microscope and superresolution microscope. miRNAs in EVs were extracted with miRNA isolation kit. Cel-39 was used as the spike-in control in qPCR. This study was approved by medical ethics committee.
Results: Profile of miRNAs in serum EVs was done with Illumina high-throughput sequencing system. 179 differentially expressed miRNAs were of statistical significance and with reads more than 30. To verify our sequencing result, EVs from TB, lung cancer and health control group were used, seven cases in each group. There were 22 miRNAs with $p < 0.01$ and change tendency in accordance with sequencing result. Further training step was performed with samples from above three group, and the sample size increased to 20 cases in each group. Only for four miRNAs was the p value smaller than 0.001. Then these four miRNAs were validated in samples from 105 TB patients, 171 lung cancer patients and 154 health control. The miR-1290 level was different among the three groups and had the highest diagnostic role between lung cancer

and TB group (area under the curve = 0.781, $p < 0.01$). In addition, the area under the curve increased to 0.96 ($p < 0.01$) when miR-1290, C-reactive protein and carcinoembryonic antigen were combined.
Conclusion: This study confirms that profiles are different between TB, lung cancer and healthy people. In these miRNAs, 4 miRNAs are differentially expressed in these three groups, and miR-1290 showed a high accuracy in differential diagnosis between lung cancer and TB patients. These results indicate that miRNAs in serum EVs possess the potential as a differential biomarker in pulmonary nodules.

PT08.11

The art of war: exosomes as carrier pigeons of the cell to protect from bacterial spread during infection with yersinia pestis

Adam Fleming[1], Heather Hobbs[1], Sherwin Parandeh[1], Valentin Giroux[2], Weidong Zhou[3], Valerie Calvert[3], Carolina Salvador-Morales[2], Nitin Agrawal[2], Emanuel Petricoin[3] and Ramin M. Hakami[4]

[1]School of Systems Biology and NCBID, George Mason University, Manassas, Virginia, USA; [2]Bioengineering Department, George Mason University, VA, USA; [3]Center for Applied Proteomics and Molecular Medicine, George Mason University, VA, USA; [4]School of Systems Biology and NCBID, George Mason University, VA, USA

Introduction: Our laboratory has been among the pioneering groups researching exosome (EX) effects during infection with highly pathogenic agents such as *Yersinia pestis* (Yp), the agent of plague. Yp is a Category A pathogen that causes high mortality and has the potential to be used for bioterrorism. There are no approved vaccines or highly effective treatments.
Methods: EXs were purified from naïve (uninfected and untreated) U937 cells (EXu) and Yp-infected U937 cells (EXi) by differential centrifugation followed by sucrose density gradient purification, and characterised by TEM and western blot analysis. Naïve monocytes were treated with EXi or EXu (as control) and analysed for effects on bacterial uptake and clearance, differentiation, and cytokine release. Proteinase K-treated EXi (PK-EXi) were also tested. Analysis of intracellular signalling events in response to EXi was performed using our protein microarray platform. EX content was also analysed using LC-MS/MS.
Results: EXi induce phenotypes in naïve monocytes that are identical to when they are infected with Yp: (a) induction of differentiation to macrophages, as indicated by a significantly prolonged G1phase of the cell cycle, increased attachment, and appearance of CD68 marker; (b) induction of significantly improved capacity for bacterial clearance; (c) significant release of the inflammatory cytokines IL-6, IL-8 and IL-10. Knockdown of IL-6 in the recipient naïve cells prior to EXi treatment abrogated EXi ability to induce increased bacterial clearance. Furthermore, PK-EXi failed to induce differentiation or IL-6 release and increased bacterial clearance, even though they are internalised by the recipient cells. Several protein pathways were identified that are strongly modulated in response to EXi, including strong activation of the p38 kinase pathway that is known to regulate IL-6 release and monocyte differentiation. Also, several EXi-associated Yp proteins were identified that are reported to have immunogenic properties.
Conclusion: Our results suggest a model in which surface proteins of the EXi prime distant naïve target cells by activating pathways such as p38 to mount immune responses similar to when they get infected, thus equipping them to fight off infection more efficiently once the Yp bacteria reach them.

PT08.12

Secretion of Toll-like receptor mRNAs via exosomes: a possible way of communicating messages against pathogens

Muhammad Nawaz[1,2], Jessica Wahlgren[1], Luisa Statello[1], Marco Maugeri[1], Alexandros Papadimitrio[1] and Hadi Valadi[1]

[1]University of Gothenburg, Gothenburg, Sweden; [2]University of Sao Paulo, Sao Paulo, Brazil

Introduction: Toll-like receptors (TLRs) are transmembrane receptors usually expressed in sentinel cells and are thought to play an important role in the recognition of invading microorganisms, such as viruses, bacteria, fungi and protozoa. During host–pathogen interactions, the microbes could penetrate into physical barriers of the first line of defence and are recognised by TLRs, which activate innate immune responses. Recent data indicate that sentinel cells secrete exosomes that may have a role in immune responses and could contribute to TLR-mediated antimicrobial defence. We hypothesised that TLR-mRNA could be packaged into exosomes during microbial infection and shuttled to other cells in order to alert neighbouring cells against pathogenic signals.
Methods: Exosomes were isolated by ultracentrifugation from bio-fluids (urine, synovial fluid) of individuals with microbial infections and from healthy controls. Exosomes were filtered using 0.22 μm filter and total RNA was isolated using miRCURY RNA isolation kit (Exiqon, DK). Differential expression of TLR-mRNA content against each infection was assessed by real time PCR. In addition, B and T-cells were stimulated *in vitro* and exosomes were examined for TLR-mRNA as compared to exosomes from non-stimulated cells.
Results: As compared to healthy subjects the TLRs 5, 6, 7 and 9 were strongly upregulated in exosomes from urine of E. coli infected patients. Wherease, TLRs 2–7 and 9 were downregulated from arthritic patient derived synovial fluid exosomes as compared to healthy individuals. TLRs in exosomes from stimulated B-cells were upregulated, whereas those from T-cells were downregulated as compared to exosomes from non-stimulated cells. This indicates the secretion of differentially expressed TLR-mRNA from infected individuals as well as stimulated cells as compared to healthy donors and non-stimulated cells.
Conclusion: Secretion of TLR-mRNA via exosomes is a potent way of communicating defensive messages against pathogen invasion. This indicates the roles of exosomes in mediating host–pathogen interactions by shuttling TLR messages which raises the possibility to use exosomes as prospective biomarkers aginsat infections. However, furthers studies would be required to elucidate underlying mechanisms.

PT08.13

Extracellular vesicles from *Staphylococcus aureus* and *Staphylococcus epidermidis* are associated with small RNA

Forugh Vazirisani[1,2], Margarita Trobos[1,2], Furqan A. Shah[1], Peter Thomsen[1,2] and Karin Ekström[1,2]

[1]Department of Biomaterials, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; [2]BIOMATCELL, VINN Excellence Centre of Biomaterials and Cell Therapy, Gothenburg, Sweden

Introduction: Delivery of bacterial components, proteins, and toxins by extracellular vesicles (EVs) from Gram-positive bacteria can affect modulation of the host immune response and cell death. However, little is known if genetic materials are associated with these EVs. The aim of the present study was to investigate whether EVs from *Staphylococcus aureus* and *S. epidermidis* contain RNA, and if so, whether the RNA content and profile differ between EVs isolated from different bacterial species and strains.
Methods: EVs were isolated from *S. aureus* ATCC 25923, *S. epidermidis* ATCC 35984, and two clinical strains *S. aureus* 64516 and *S. epidermidis* 64518 isolated from osteomyelitis associated with orthopaedic implants, by filtration and centrifugation steps. EVs were characterised by Western blot, Nanosight and electron microscopy. RNA was extracted, detected and quantified by Ribogreen fluorescence and Bioanalyzer. RNase treatment was used to confirm that the detected nucleotides were RNA and not DNA.
Results: All staphylococcal strains released EVs with a size of ~100 nm. Protein A, SCP-A, α- and δ-toxins were detected in *S. aureus* EVs while *S. epidermidis* EVs contained only δ-toxin. The *S. epidermidis* 35984 released a higher number of EVs than *S. aureus* 25923 (50.9×10^8/ml and 6.1×10^8/ml, respectively). RNA was detected in EVs from all strains. The electropherograms showed that *S. aureus* 25923 and *S. epidermidis* 35984 EVs contained mainly small RNA, while the EVs from clinical strains also contained ribosomal RNA peaks. RNase treatment removed most of the nucleotides, supporting the finding that the EVs contain RNA. A higher amount of EV RNA was obtained from the clinical strains compared with ATCC strains, and from EVs isolated from *S. epidermidis* compared to *S. aureus.*
Conclusion: EVs from Gram-positive bacteria, and in particular staphylococci, contain RNA. The knowledge of the molecular content of EVs is of importance to understand the mechanisms of EV function.

PT08.14

Membrane vesicle subpopulations in *Escherichia coli* UPEC: a methodological comparison

Priscila Dauros-Singorenko[1], Alana Whitcombe[1], Vanessa Chang[2], Denis Simonov[3], Anthony Phillips[1,3], Simon Swift[2] and Cherie Blenkiron[1,2]

[1]School of Biological Sciences, University of Auckland, Auckland, NZ; [2]Department of Molecular Medicine and Pathology, University of Auckland, Auckland, NZ; [3]Department of Surgery, University of Auckland, Auckland, NZ

Introduction: Formation of membrane vesicles (MVs) in bacteria is now found to be a common but still understudied process. These MVs have shown to be a heterogeneous population, carry diverse cargos and have different biological roles in an infectious disease scenario. The isolation and purification method is critical in interpreting meaningful results and understanding MV functionality in the disease, but is lacking standardised protocols or guidelines in the prokaryotic field. Here, we compare standard purification method density gradient centrifugation (DGC) method with alternative labour, cost and time effective method of size exclusion chromatography (SEC).
Methods: "Crude" MVs preparations from independent *Escherichia coli* Uropathogenic 536 cultures were used for fractionation with DGC ($N = 3$) or SEC ($N = 3$). Molecules (particles, protein, RNA, LPS) of interest were quantified in resulting fractions. Characterisation of fractions was also done by polyacrylamide gel electrophoresis (PAGE) and electron microscopy (EM).
Results: MV preparations separated by DGC consistently generated six fractions/layers, whereas second and third lightest density fractions contained most of particles (96%), protein (94%), LPS (94%) and RNA (91%). There were no differences in quantified molecules, protein profiling and microscopic analysis between these two DGC vesicle-enriched fractions. EM revealed single membrane structured vesicles only in fractions 2 and 3. On the other hand, 14 fractions were arbitrarily collected by SEC method. Fractions 8, 9, 10 and 11 contained most (>84%) of particles (90%), protein (90%), LPS (66%) and RNA (86%). All analysed molecules, except for LPS, in these fractions constantly decreased as their elution times increased. Differences in MV crude load affected SEC fractionation; higher MV loads shifted vesicle-enriched fractions to later eluted fractions. EM also showed well structured vesicles in vesicle-enriched fractions along with the presence of flagella aggregates.
Conclusion: Depending on the vesicle molecule/population of interest, SEC can be an effective, highly reproducible and quick alternative method for *E. coli*'s MV purification. Attention is recommended to MV crude load optimisation prior definitive use on samples.

PT08.15

Qualitative changes in the proteome of milk-derived extracellular vesicles during induced staphylococcus aureus mastitis

Zuzana Krupova[1,2], Anne Chaize[1], Natayme Rocha[3], Christine Péchoux[1], Celine Henry[4], Pierre Defrenaix[2], Yves Le Loir[3] and Patrice Martin[1]

[1]GABI, INRA, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France; [2]EXCILONE, Elancourt, France; [3]STLO, INRA, Rennes, France; [4]INRA UMR1319 MICALIS, PAPPSO, Jouy-en-Josas, France

Introduction: Mastitis affects the hygienic quality of milk and its composition, leading to production losses, payment penalties and additional costs associated with antibiotic treatments. Little is known about changes in extracellular vesicle (EV) composition and contribution to mastitis development in regard to host immune response. *S. aureus* also releases EVs which are known to play a role in delivery of toxins, enzymes and other structural molecules to host cell, modulating the host immune response. Our project is focusing on the host response induced by an experimental *S. aureus* infection to sensitive and resistant goats selected on the basis of somatic cell counts (SCC) in milk. The objective of this study is to precisely assess the impact of *S. aureus*-induced mastitis on EVs secretion and to characterise the components that underlie the resistance to mammary infection.
Methods: Goats divergently selected according to SCCs in milk were experimentally infected with *S. aureus* 122.25 strain. Milk was sampled before and after inoculation of a half-udder at several time-points after infection. Milk-derived EVs and *S. aureus*-derived EVs isolated from culture supernatants were purified using a sucrose gradient ultracentrifugation method. EVs were validated by TEM, exosome specific protein markers were detected by WB and the size distribution and particle concentration were measured by TRPS and NTA. The proteome was acquired by LC-MS/MS.
Results: The average size of EVs purified from culture supernatant of *S. aureus* was 113 ± 55.8 nm with particle concentration of 4.59E +5/mL culture supernatant. Due to proteolysis of the major milk proteins after infection, an optimisation of the milk-derived EV purification method was necessary. The milk-derived EV concentration and size distribution varied along the infection. A total of 74 proteins were identified in the EVs of 122.25 *S. aureus* strain. More than 205 proteins were identified in milk-derived EVs before induced infection with important variation in protein composition after bacterial challenge. Several virulence-associated proteins are found in the *S. aureus* EVs and can be detected in the infected milk-derived EVs.

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Poster Session PT09 – EVs in Diseases of the Central Nervous System
Chairs: Lesley Cheng and TBD 5:15–6:30 p.m.

PT09.01

Serum exosome miRNA profiles have the potential to diagnose and predict disease stage in multiple sclerosis

Saeideh Ebrahimkhani[1], Fatemeh Vafaee[2,3], Paul Young[4], Michael Barrnet[1], Catherine Suter[4] and Michael Buckland[1]

[1]Sydney Medical School, Brain and Mind Centre, The University of Sydney, Sydney, NSW, Australia; [2]Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia; [3]School of Mathematics and Statistics, The University of Sydney, Sydney, NSW, Australia; [4]Victor Chang Cardiac Research Institute, South Wales, Australia

Introduction: Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) and typically strikes young adults, disproportionally women. There is currently no one definitive test for MS. Diagnosis, and disease activity monitoring is based on clinical examination, MRI, CSF studies, and neurophysiology, but these are associated with high costs and limited accessibility. Therefore, blood-based biomarkers for MS are urgently needed. We hypothesise that selective package of small RNA in serum-derived exosomes can be developed into a blood-based assay for MS detection and monitoring.
Methods: In this study we profiled exosome-borne sncRNAs from MS patient serum samples in different disease courses and also a subtype of MS patients (relapsing–remitting multiple sclerosis, RRMS) in four-time points (two years), along with matched controls using high-throughput sequencing. Furthermore, we used sophisticated bioinformatics approaches to refine the predictability power of identified miRNAs.
Results: We reported that MS patient sera exhibit dysregulation of miRNAs in relation to controls and that the panel of such miRNAs shows specificity to the disease subtype. Importantly, we have also identified a group of miRNAs that are associated with MS progression from RRMS to S/PPMS.
Conclusion: This study shows that serum exosomes from MS patients are meaningfully altered in their miRNA profiles, which can potentially be utilised as biomarkers. To our knowledge, this is the first proof-of-principle demonstration that miRNAs from serum exosomes can be used to distinguish stages of MS in patients.

PT09.02

Systems-modelling and biological evidence for alteration of extracellular vesicles in Huntington's disease

Francesca Farina[1], François-Xavier Lejeune[1], Frédéric Parmentier[1], Jessica Voisin[1], Satish Sasidharan Nair[1], Clotilde Théry[2] and Christian Neri[1]

[1]Institut de Biologie Paris-Seine (IBPS), CNRS UMR 8256, Paris, France; [2]Institut Curie, PSL Research University, INSERM U932, Paris, France

Intercellular communication mediated by extracellular vesicles (EVs) is emerging as a mechanism that is important to neuronal homeostasis and integrity. However, there is little information available on the importance of EV signalling in response to proteotoxic stress in Huntington's disease (HD). Using network methods to integrate HD gene expression datasets, we reconstructed a computational model of the transition from the early (cell differentiation) to intermediate (dysfunctional striatum) and late (advanced neurodegeneration) phases of the HD process. This model indicates that gene deregulation in HD could impact on EV signalling across biological phases of the disease. To test for this hypothesis, we analysed EVs in striatal cells derived from HD knock-in mice. In studies of EVs, it is important to discriminate different subtypes of EVs based for instance on vesicular size as this may determine function. In these experiments, we used protocols and EV markers that allow for differential analysis of EV subtypes to be performed, testing for changes in secreted amount and protein cargo composition. The results suggest that EV subtypes may be altered in cells expressing mutant huntingtin.

PT09.03

The ratio between oligomer to monomer amyloid beta in neuronal exosome extracted from plasma discriminates Alzheimer's patient from normal control

Kyeong-sik Shin[1], Jae Hoon Ji[2], Seong-chan Jun[3] and Ji Yoon Kang[2]

[1]Cantis; [2]KIST, Seoul, Republic of Korea; [3]Yonsei University, Seoul, Republic of Korea

Introduction: Amyloid beta oligomer has been considered as a biomarker of Alzheimer's disease (AD) but it is hard to quantify the concentration due to its diverse forms in blood, much low concentration and lack of specific antibody. Hence, this paper suggests 'the oligomer to monomer ratio of amyloid beta in neuronal exosome' as a new biomarker and validate it with electrochemical biosensor.
Methods: Plasma samples were processed with ExoQuick and agarose gel to extract neuronal exosome. The samples were diluted by 4 times with a repeated factor of 5, and the impedance of sensor was measured for each diluted sample. The slope with respect to dilution factors (1/5, 1/25, 1/125) was used to calculate the ratio based on the slope of sensor signal with respect to dilution factors since the sensor's impedance is proportional to the size of detected molecules. The sensor was bead-based electrochemical impedance spectrometry (BEIS) sensor comprising of two electrodes, microwell array and permanent magnet. The magnetic beads coated by capture antibody were incubated with neuronal exosomes and trapped in each microwell by a magnetic bar.
Results: The plasmas of patients and normal control were collected at SNUBH (AD:25, NC: 21). The ratios of AD patients were almost perfectly discriminated from that of NC (normal control) with the sensitivity of 100% and the selectivity of 100%.
Conclusion: The oligomer to monomer ratio of amyloid beta measured by BEIS sensor was demonstrated to be a valuable biomarker to disseminate AD from NC. The reliability of diagnosis will be validated by additional testing with multi-centre samples.

PT09.04

Neuroprotective mechanisms of extracellular small heat shock proteins in neuroinflammation

Joy I. Irobi

Hasselt University, Hasselt, Belgium

Heat shock binding proteins (HSPB) provide protection from cellular and environmental stress factors as molecular chaperones to keep protein homeostasis. Extracellular or membrane-bound HSBP have a protective role in mediating immunological functions and immunomodulatory activity. Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system, featured by immune cell mediated destruction of the insulating myelin around neuronal processes. Previously we showed that small heat shock binding proteins (HSPB1 and HSPB8) have critical neuroprotective functions in the peripheral nervous system where mutations in these molecular chaperones cause peripheral neuropathy and neuronal death. We showed that expression of mutant HSPB1 decreased acetylated α-tubulin abundance and induced severe axonal transport deficits. HSPB have pleiotropic cytoprotective functions and interacts with diverse key molecular partners. HSPB5 was identified as candidate autoantigen in MS. HSPB are induced during MS lesion development and are found in the blood of MS patients, peaking during relapses.
Intracellular HSPB are released out and their potential extracellular functions during neuroinflammation have not been studied extensively. Interestingly HSPB are expressed in brain glial cells known to secrete exosomes or extracellular vesicles expressing HSPB. Exosomes are nanovesicles which are of great importance for their biomarker potential in

disease diagnosis and therapy. We hypothesise that dysfunctional trophic support of HSPB in transcellular exosome signalling during neuroinflammation could result in deficits in the remyelination repair process. Investigating the extracellular signalling of released HSPB in response to local brain inflammation and understanding the HSPB-exosome-mediated uptake in brain glial cells, could offer key molecular targets on how this process may be harnessed for remyelination strategies.

PT09.05

Extracellular vesicles as regulators of inflammation in ischemic stroke

Nea Bister[1], Paula Korhonen[1], Henna Konttinen[1], Nikita Mikhailov[1], Sanna Loppi[1], Laura J. Vella[2], Andrew F. Hill[3], Katja Kanninen[1], Rashid Giniatullin[1] and Tarja Malm[1]

[1]A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland; [2]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Victoria, Australia; [3]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Victoria, 3084, Australia

Introduction: Extracellular vesicles (EVs), such as exosomes, microvesicles and apoptotic bodies, are released to the body fluids by all cell types. EVs have shown to be taken up by recipient cells in which their cargo can modulate cellular functions. Altered vesicle secretion has been implicated in several pathological conditions, including neurodegenerative disorders such as Alzheimer's disease. However, the effect of ischemic stroke on EV secretion is completely unknown. Continuously failing clinical trials suggest that pathological mechanisms of stroke are still poorly understood. As EVs are appreciated as important players in cell-to-cell communication, and stroke is well known of its progressive pathology and associated neuroinflammation, it is likely that EVs play a role in stroke pathology.
Methods: The aim of this study was to investigate whether ischemic stroke alters the secretion of EVs in the brain. Mice were subjected to permanent middle cerebral artery occlusion after which the brains were collected and EVs isolated by sucrose density gradient ultracentrifugation. The morphology and size distribution of EV preparations were characterised by transmission electron microscopy and nanoparticle tracking analysis (NTA), respectively. In addition, NTA was used to determine the EV concentration of the samples. The impact of EVs on microglial viability and cytokine secretion was evaluated by MTT assay and cytokine bead assay, respectively.
Results: Ischemic stroke increases the amount of EVs in the brain tissue at 2 h post-surgery. Brain derived EVs increase microglial mitochondrial activity but do not alter the activity of neurons. However, at 12 h post-stroke this effect is lost also in microglia, suggesting cell specific and time dependent changes in the cellular impact of EVs after stroke.
Conclusion: This preliminary data suggets that EVs may have a role in stroke pathology. Further studies are needed to characterise molecular composition of EVs, leading to better understanding of the specific mechanisms of EVs and their relevance in stroke.

PT09.06

Flow cytometry analysis of blood microvesicles in patients with multiple sclerosis

Jakub Soukup[1,2], Marie Kostelanska[1], Eva Havrdova[3] and Karel Holada[1]

[1]Institute of Immunology and Microbiology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic; [2]Department of Genetics and Microbiology, Faculty of Science, Charles University, Prague, Czech Republic; [3]Department of Neurology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

Introduction: Several studies reported elevated numbers of diverse cellular microvesicles (MVs) in blood of patients with Multiple Sclerosis (MS). To explore the diagnostic potential of MVs in MS we utilised flow cytometry for simultaneous analysis of platelet, erythrocyte, B-cell, T-cell and endothelial MVs.
Methods: Blood of MS patients in exacerbation of the disease (n = 16) or healthy controls (n = 16) was collected in K_2EDTA and processed within 20 minutes. MVs were isolated from platelet free plasma (14,000g, 70 min), washed with PBS-BSA and incubated with antibody CD105, CD235a, Annexin V or with combination of antibodies (CD41+CD36) or (CD45+CD19+CD3) to distinguish MVs derived from different cells. Labelled MVs were immediately analysed on BD FACSCanto II flow cytometer. The vesicles were divided by size in 3 groups using ApogeeMix beads: >1.2 μm, 0.5–1.2 μm (MVs gate) and <0.5 μm.
Results: Relative number of endothelial (CD105+) MVs was higher in healthy controls (HC) than in MS patients (7.6% vs. 4.5%, p = 0.0098). Similarly, also relative number of B-cell (CD19+) and T-cell (CD3+) MVs was higher in HC than in MS patients, 6.7% vs. 3.4% (p = 0.0268) and 14.3% vs. 6.9% (p = 0.0037), respectively. The differences in the rest of analysed populations of MVs were not statistically significant as were not the counts of MVs/μl of plasma. In plasma deprived of MVs (supernatant after 14,000g, 70 min) remained particles positive for the selected markers, but on contrary analysis of these MVs suggested more Annexin V+ MVs in MS patients 260 MVs/μl vs. HC 175 MVs/μl (p = 0.0249).
Conclusion: The analysis of washed plasma MVs did not reproduce previously published results demonstrating higher counts of non-washed platelet or endothelial MVs in blood plasma of MS patients. In contrast, relative numbers of T-cell, B-cell and endothelial MVs were lower than in HC demonstrating critical effect of sample preparation on the results of MVs analysis.

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PT09.07

Enrichment of non-coding RNA-species in exosomes: potential biomarkers for Alzheimer's disease

Rhodri Thomas[1], Elisa Majounie[1], Rebecca Sims[1], Juan M. Falcón-Pérez[2], Aled Clayton[3] and Julie Williams[1]

[1]Cardiff University, Cardiff, United Kingdom; [2]CIC bioGUNE; [3]Division of Cancer and Genetics, School of Medicine, Cardiff University and Velindre Cancer Centre, Cardiff, United Kingdom

Introduction: Identifying exosomal RNA as biomarkers of disease is a growing field of research, yet there is little known about the relationship between this vesicular RNA cargo and the RNA present in the cell of origin. Past studies have often used small RNA sequencing approaches, which pre-selects for a subset of smaller length transcripts, as opposed to total RNA.
Methods: Next-generation total RNA sequencing was performed comparing total cellular and total exosomal RNA extracted from a neuroglioma cell-line, with only the ribosomal RNA depleted. Exosomes were isolated by ultracentrifugation at 200,000g for 2 h followed by washing with PBS and a second ultracentrifugation to pellet. These were thoroughly characterised by nanoparticle tracking analysis, cryo-electron microscopy, sucrose density gradient, western blot and enzyme-linked immunosorbent assay.
Results: Exosomal RNA is dramatically distinct from source cell RNA. Only 1% of the exosomal RNA mapped bases resided in exonic regions of the human genome compared to 40% with the cellular RNA. Rather the majority of exosomal RNA was intronic and intergenic. Further analysis revealed 1554 long non-coding RNAs, which passed Bonferroni correction for multiple testing, that were differentially expressed between cells and exosomes.
Conclusion: The constitution of RNA in exosomes is distinct from source cells and they may act as a repository for precursor-messenger RNA and other untranslated species. This suggests that biomarkers of disease that have previously been identified in cells is unlikely to correlate with what is detectable in exosomes. This highlights the potential of discovering new biomarkers of Alzheimer's or other diseases that lie in the non-coding genome, and suggests that the pursuit of biomarker discovery in exosomes could be a fruitful avenue of research.

PT09.08

Cell-type specific exosome signalling and disease propagation in ALS

Eoin D. Brown[1], Ming Sum Chiang[1], Julia Yelick[2] and Yongjie Yang[1]

[1]Department of Neuroscience, Tufts University, MA, USA; [2]Tufts University, MA, USA

Introduction: Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disorder characterised by the degradation and subsequent death of motor neurons in the spinal cord and motor cortex. The mechanisms responsible for ALS propagation are not yet fully understood, but are likely to involve the transmission of disease associated proteins and other toxic factors. Emerging evidence from our group and current literature has provided evidence that exosomes play an important role in facilitating the pathology of ALS and other neurodegenerative diseases. Therefore, it is crucial to understand the *in vivo* characteristics, distribution and pathological behaviour of exosomes within the CNS. To enable this investigation, we have developed a novel Cre-dependent CD63 exosome reporter mouse to allow cell specific GFP labelling of endogenous exosomes *in vivo*.
Methods: Our model utilises the Cre-Lox recombination system, featuring a floxed stop codon upstream of copGFP tagged CD63, which labels CD63 expressing exosomes in a cell specific manner when induced with promoter driven Cre recombinase.
Results: To validate the system, we stereotactically injected the cortex of copGFP-CD63/Ai14-tdt mice with AAV8-CAMKII-cre or AAV5-GFAP-cre, with GFP expressing puncta being observed in a cell specific manner. These puncta were detected both intracellularly and extracellularly of the parent cell (as visualised by Cre-activated Ai14-tdt expression). The identity of the copGFP/CD63 puncta as exosomes was confirmed with immunohistochemical staining against common exosome markers. Using this model, we observed cortical neurons to secrete a more abundant population of exosomes that migrate to a further degree than astrocyte exosomes. Comparison of small RNA content in primary cultured neurons and astrocytes show that small RNA populations are enriched in neuronal exosomes, as compared to astrocytes exosomes.
Summary: In summary, we have developed and validated a novel mouse model that enables the cell-specific labelling of endogenous exosomes by expressing copGFP-CD63. This system offers a new and invaluable tool that will prove key in deciphering exosome biogenesis, cargo loading, recipient cell processing, and the role exosomes play throughout development and disease propagation.

PT09.09

Increased miR-124 cargo in circulating extracellular vesicles after experimental traumatic brain injury

Jenni Karttunen[1], Vicente Navarro Ferrandis[1], Mette Heiskanen[1], Kirsi Rilla[2], Arto Koistinen[3], Shalini Das Gupta[1], Niina Vuokila[1], Noora Puhakka[1], David J. Poulsen[4] and Asla Pitkänen[1]

[1]University of Eastern Finland, A.I. Virtanen Institute for Molecular Sciences, Department of Neurobiology, Joensuu, Finland; [2]Faculty of Health Sciences, School of Medicine, Institute of Biomedicine, University of Eastern Finland, Joensuu, Finland; [3]SIB labs, University of Eastern Finland, Joensuu, Finland; [4]University at Buffalo, The State University of New York, School of Medicine and Biomedical Sciences, NY, USA

Introduction: Traumatic brain injury (TBI) is a worldwide problem with ~10 million new cases annually. Impact-induced primary injury after TBI occurs within seconds to minutes. Post-TBI secondary brain pathologies progress for weeks to months, and worsen the evolution of comorbidities. Extracellular vesicles (EVs) have recently been recognised as mediators of intercellular communication. However, little is known about their contribution to the evolution of post-TBI secondary damage or recovery. We assessed the characteristics of plasma EVs and their contents of brain-enriched miR-124-3p during the first week post-TBI. We also tested whether EV miR-124-3p levels would serve as biomarkers for TBI diagnosis.
Methods: Adult male rats were subjected to lateral fluid-percussion injury. Trunk plasma was collected at 2 or 7 d post-TBI. Naïve and sham-operated animals served as controls. EVs were isolated from plasma using commercial kit based on membrane particle precipitation. The purification method was evaluated using nanoparticle tracking analysis (NTA), scanning electron microscopy, and western blot. The number and size distribution of plasma EVs after TBI were measured with NTA. miR-124-3p concentration was measured from isolated EV-RNA with quantitative PCR. Gene set enrichment analysis (GSEA) was conducted for three EV related gene sets using available mRNA-seq (3 month post-TBI) and microarray (32 h post-TBI) data from brain tissue as rank lists.
Results: NTA showed a decrease in the number of plasma EVs at 2 d and 7 d post-TBI. GSEA revealed transcriptomic-level enrichment of gene sets related to EVs, especially in the perilesional cortex. The level of plasma EV miR-124-3p concentration was increased a 2 d post-TBI as compared to controls or 7 d post-TBI samples. Receiver operating characteristic analysis indicated that plasma EV miR-124 level differentiated TBI animals from controls (AUC 0.922, $p < 0.05$)
Conclusion: Our data demonstrate dynamic changes in the number of plasma EVs, regulation of genes related to EV production in the brain, and regulation of plasma EV contents of brain-enriched miR-124-3p during the first week post-TBI.

PT09.10

Adherent proteins may account for some of the bioactivity of small extracellular vesicles (exosomes) secreted by mesenchymal stem/stromal cells (MSCs)

Dong-Ki Kim[1], Hidetaka Nishida[2], Su Yeon An[1], Eun Hye Bae[1], Ashok K. Shetty[1,3] and Darwin J. Prockop[1]

[1]Institute for Regenerative Medicine, Texas A&M University College of Medicine, College Station, TX, USA; [2]Joint Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University; [3]Olin E. Teague Veterans' Medical Center, Temple, TX, USA

We recently developed a protocol for chromatographically isolating small extracellular vesicles from the culture media of human mesenchymal stem/stromal cells (hMSCs). The vesicles lack a series of epitopes found on hMSCs, are CD9-CD63+CD81+, are about 100 nm in diameter, and have anti-inflammatory properties. Therefore we have referred to them as A1-exosomes. In a mouse model of traumatic brain injury, a single intravenous administration of A1-exosomes decreased brain inflammation after 12 h and rescued behavioural deficits present in controls after about 1 month (1). Proteomic analysis of the A1-exosomes by HPLC/MS/MS indicated the presence of over 100 proteins, about a third of which were secreted factors, plasma membrane ligands, or matrix proteins. SDS-gel assays after tryptic digestion confirmed that a large fraction of the proteins were extracellular. Further fractionation of the A1-exosomes by chromatography generated two peaks that differed in their protein profiles. The results indicated that exosomes secreted by MSCs contain a large number of adherent proteins that may account for some of their biological activities.

Funding: Supported in part by NIH grant P40OD11050.

Reference

1. Kim et al., *Proc Natl Acad Sci USA*. 2016; 113: 170–175.

LBP.20

Neuroprotective mechanisms of extracellular small heat shock proteins (HSPB1 and HSPB8): The role of HSPB in transcellular EV signaling in neuroinflammation

Joy I. Irobi[1], Joel Beaumont[2], Simona Cecchi[2], Vincent Timmerman[3] and Luc Michiels[1]

[1]Hasselt University, Biomedical research institute, Martelarenlaan 42, 3500 Hasselt, Belgium; [2]Hasselt University, Hasselt, Belgium; [3]Antwerp University, Antwerp, Belgium

Introduction: Multiple sclerosis (MS) is a chronic autoimmune disease affecting the central nervous system. The repair mechanism of MS is still

unknown but small heat-shock proteins (HSPBs) have been shown to be upregulated in the blood of MS patients. We showed that mutations in HSPB1 and HSPB8 caused peripheral neurodegeneration commonly known as Charcot-Marie-Tooth (CMT) disease. The HSPB1 and HSPB8 genes are ubiquitously expressed and have vital function in preventing axonal damage. In addition, skin fibroblasts of CMT patients exhibit HSPB8 protein aggregates indicating defects in HSPBs chaperoning activity. Although the intracellular role of HSPBs has been proven, the extracellular functions remain unclear. One way that HSPBs are released into the extracellular space is though extracellular vesicles (EV). Neural cells release EVs either carrying beneficial or detrimental biomarkers into the environment. We study the protective activities in early inflammation and use extracellular vesicles expressing HSPB8 complexes as a delivery vehicle.
Methods: The effect of inflammation on the protective mechanisms of EV-HSPBs is investigated. We will: 1) Establish EV-HSPBs expressing stable cell lines for the production of EV-rich conditioned medium (CM). 2) Isolation, purification and characterization of EV-HSPB (normal and inflamed EV-HSPB8). 3) Measuring the survival and chaperone activity of neural cells stimulated with nEV-HSPB8 and iEV-HSPB8.
Results: Our pilot study shows that in early inflammation (24h), there is an upregulation of total EV RNA including microRNA and mRNA in inflammation triggered cells. Our results also show a downregulation of HSPBs mRNA levels in TNF-α stimulated microglial and oligodendrocyte cells. These observations in early inflammation of an upregulation of total EV RNA and a downregulation of HSPB1 and HSPB8 in neural cells together with the lack of neuroprotection observed in chronic inflammation support the hypothesis that there is an impairment in the molecular chaperoning and cytoprotective activities
Summary/Conclusion: Understanding the extracellular function of EV loaded with HSPB8 chaperoning activities in neuroinflammation will provide new insights to advance the development of MS therapeutic strategies
Funding: This work was financed by FWO travel grant, Hasselt University and Trans Tech Diagnostics project.

LBP.21

Withdrawn at author's request.

LBP.22

Reactive astrocytes-derived exosomes promote neurogenesis through Wnt signaling: implication for Parkinson's disease therapy

Lu Yang, Fei Qu and Min Zheng

University of Electronic Science and Technology of China, Sichuan, China

Introduction: The phenomenon of adult dopaminergic neurogenesis intrinsic to the dopamine-depleted striatum has been indicated in Parkinson's diseaseï¼ˆPDï¼‰. However, the underlying mechanisms are not completely clarified. Reactive astrocytes exhibit their ability to influence neuroprotection, including regulation of neurogenesis through Wnt signaling in PD. Currently, exosome has merged as a novel mediator for cell-cell communication. Importantly, recent evidence indicated that exosomal Wnt proteins are involved in multiple processes including angiogenesis and development. Thus, we hypothesize that exosomal Wnt derived from reactive striatal astrocytes play a role in regulating the dopaminergic neurogenesis in PD.
Methods: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was used to induce astrogliosis *in vitro* and *in vivo*. Exosomes were collected by ultracentrifugation and assessed by EM and Nanosight. Wnt-containing exosome mediated neurogenesis was examined by CyQuant assay & BrdU labeling.
Results: In our study, we detected a significant increase of Wnt expression in both MPTP-pretreated striatal astrocytes as well as their released exosomes. Wnt-enriched exosomes could be taken up by the NSCs, resulting in increased proliferation of NSCs. This effect was inhibited by either pre-treating astrocytes with exosome inhibitors or siRNA-mediated knocking down of Wnt gene in astrocytes. Our result also revealed that the canonical Wnt signaling was activated while the NSCs were exposed to those exosomes. Our *In vivo* study further demonstrated the effect of Wnt-containing exosomes in promoting neurogenesis in the striatum of MPTP-lesioned mouse model.
Summary/Conclusion: We conclude that exosomal Wnt were released from MPTP-activated striatal astocytes, which activate the canonical Wnt signaling pathway in the NSCs and can be used to promote neurogenesis both *in vitro* and *in vivo*. Our studies reveal novel regulatory strategies of neurogenesis in PD, indicating the therapeutic potential of Wnt-containing exosomes in PD.
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LBP.23

Fast or slow moving in ALS patients: Role of immune MVs in neuroinflammation

Sabrina La Salvia[1], Uta Erdbruegger[2], Luca Musante[3], Daisy Sproviero[4], Marta Giannini[4], Susanna Zucca[5], Orietta Pansarasa[4], Mauro Ceroni[6], Joanne Lannigan[7] and Cristina Cereda[4]

[1]Genomic and post-Genomic Center, IRCCS, C. Mondino National Institute of Neurology Foundation; [2]Department of Medicine/Nephrology Division, University of Virginia, VA, USA; [3]Department of Medicine, Nephrology Division, University of Virginia, VA, USA; [4]Genomic and Post-Genomic Center, IRCCS, C. Mondino National Institute of Neurology Foundation, Pavia, Italy; [5]Genomic and Post-Genomic Center, National Institute of Neurology Foundation, Pavia, Italy; [6]Neurology Department, National Institute of Neurology Foundation, Pavia, Italy; [7]School of Medicine, Flow Cytometry Core, University of Virginia, VA, USA

Introduction: Amyotrophic lateral sclerosis (ALS) is an idiopathic, fatal neurodegenerative disease of the human motor system. Immune responses from active T cells play likely an important pathogenic role. However, there is no particular diagnostic biomarkers available. Microvesicles (MVs) are candidate biomarkers. An improved understanding of MVs biological processes in ALS will help to define markers related to the progression of the disease and contributing to future therapeutic nanovesicles. The aim of our study was to characterize MVs as novel biomarkers in plasma of ALS patients by Imaging Flow Cytometry (IFC) focusing on MVs deriving from immune cells.
Methods: MVs from 20 ALS patients and 20 healthy volunteers were obtained by differential centrifugation from platelet poor plasma (1000g for 10 minutes, 1600g for 20 minutes). Enumeration and phenotyping was performed with IFC (AmnisImage-StreamX Mark II) using the following markers: CD4, CD8, CD25, CD45RO and CD45RA; Annexin V (AnnV) and Calcein were used as general MV membrane markers. MVs concentration was measured with qNano gold with a NP400 nm.
Results: There is no difference in concentration and size distribution of MVs between ALS and controls. In addition, no difference in numbers of AnnV+ and AnnV- MVs was found. However, we observed high levels of CD4+/CD25+/AnnV+ MVs in plasma samples from ALS patients (MVs 2,5 particles/ul; p=0.04) compared to controls (Mvs 0,9 particles/ul.) There is also a difference between fast and slow ALS patients using CD45RA/AnnV+ (1,0 particles/ul in fast vs 5,4 particles/ul in slow ALS; p=0.02) and CD45RO/AnnV+ (25,2 particles/ul fast vs 9,1 particles/ul slow; p=0.036).
Summary/Conclusion: Imaging flow cytometry is a new sensitive tool to provide comprehensive phenotyping of MV origin in ALS. We found high levels of CD4+/CD25+/AnnV+ T-regulatory cells (Tregs) MVs in our cohort of ALS patients. This supports previous finding that Tregs are neuroprotective in ALS (Henkel, 2013). In addition, our data indicates that MVs of ALS patients carry markers of naive T lymphocytes (CD45RA) and activated and memory T lymphocytes (CD45RO). Further studies are needed to understand this new phenotype of CD45RA/AnnV+ and CD45RO/AnnV+ MVs in the slow and fast progression group of ALS patients. This finding might indicate different steps of T cell activation.

LBP.24

Cerebrospinal fluid exosomal small RNA profiling by next-generation sequencing

Yohsuke Yagi and Takanori Yokota

Tokyo Medical and Dental University, Tokyo, Japan

Introduction: MicroRNAs (miRNAs), especially those contained in human body fluids, have been reported as potential biomarkers. Among various body fluids, the cerebrospinal fluid (CSF) is an important profiling target for diagnosis and monitoring of various neurological diseases. However, relevant genome-scale studies are limited and no studies have profiled exosomal miRNAs in CSF. Therefore, we conducted a next-generation sequencing-based genome-wide survey of small RNAs in the exosomal and non-exosomal (supernatant) fractions of healthy human CSF as well as serum in each donor.
Methods: The first batch of samples was derived from three donors and subjected to a genome-wide NGS-based survey. CSF samples were obtained via lumbar puncture and corresponding serum samples were simultaneously isolated from peripheral blood. Samples were further divided into exosomal and supernatant fractions via the ultracentrifugation method. Seven milliliters of CSF and 3 ml of serum from each donor were subjected to ultracentrifugation. Total RNA was isolated from each individual fractionated sample and subjected to a NGS analysis. The second batch of samples from three additional donors was subjected to focused validation via digital PCR (dPCR).
Results: MiRNA was enriched in the exosomal fractions relative to the supernatant fractions of both CSF and serum. We also observed substantial differences in exosomal miRNA profiles between CSF and serum. Half of the reported brain miRNAs were found in CSF exosomal fractions and the majority (97.7%) of miRNAs detected in CSF exosomes were reported to be expressed in brain tissue. Our data suggest that the brain is a major source of CSF exosomal miRNAs. In particular, miR-1911-5p, specifically expressed in brain tissue, was detected in CSF but not in serum, as confirmed by dPCR.
Summary/Conclusion: Here we provide the important evidence that exosomal miRNAs in CSF may reflect brain pathophysiology.

Poster Session PT10 – EVs in Tumour Metastasis and Angiogenesis
Chairs: Takahiro Ochiya and Simone Principe 5:15–6:30 p.m.

PT10.01

Cholangiocarcinoma exosomes: proteomic insights and plausible role in carcinogenesis

Suman Dutta and Arthit Chairoungdua

Mahidol University, Salaya, Thailand

Introduction: Cholangiocarcinoma (CCA), a severe malignant tumour of bile duct epithelia, is highly prevalent in Asian countries and is unresponsive to chemotherapeutic agents. Thus, a novel biological entity with high target specificity for early diagnosis and treatment are urgently needed. Exosomes are small membrane-bound vesicles found in biological body fluids, released by most cell types including cancer cells. Exosome contains cell and cell state specific subset of proteins and nucleic acids corresponding to particular cell types and play essential roles in pathophysiological events. The present study aimed to identify biomarkers in exosome released by CCA cells and to assess their cargo contents in the development and progression of CCA.
Methods: Sequential centrifugation and ultrafiltration were used to isolate exosomes from CCA cells. derived from patients. The exosomes were characterised by TEM and western blot with marker antibodies. PKH67 linker-dye was used for uptake assay. Matrigel chambers were used for migration and invasion analysis. Confocal microscopy was employed for protein localisation and Nano LC-MS/MS was used to identify proteins. Ingenuity pathway and gene ontology analysis tool were used to categorise protein class and to predict underline molecular pathways.
Results: Upon incubation, exosomes were internalised into H69 cholangiocytes and had no effects either on viability or proliferation of the host cells. Interestingly, only the exosomes from KKU-M213 cells, isolated from the most aggressive form of CCA cells, induced migration and invasion of H69 cells. Proteomic analysis, by nano LC-MS, of the exosomes from KKU-M213 cells, disclosed multiple cancer-related proteins that were absent in H69 exosomes. On the other hand, a few proteins observed in H69 cell-derived exosomes were absent in KKU-M213 exosomes. Consistent with the proteomic profile, treatment with KKU-M213 exosomes induced β-catenin and reduced E-cadherin expressions in H69 cells.
Conclusion: Collectively our results suggest a direct cell-to-cell transfer of oncogenic proteins via the exosomal pathway that plays a pivotal role in CCA pathogenesis. The identified exosomal cargo may serve as a composite biomarker for early detection of the disease and thus might enlighten new treatment strategies.

PT10.02

Functional roles of CaF-derived extracellular vesicles in scirrhous type gastric cancer

Yutaka Naito[1], Masakazu Yashiro[2], Kosei Hirakawa[2], Tohru Kiyono[3], Wataru Yasui[5] and Takahiro Ochiya[1]

[1]Division of Molecular and Cellular Medicine, National Cancer Centre Research Institute, Japan; [2]Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Japan, Osaka, Japan; [3]Division of Carcinogenesis and Cancer Prevention, National Cancer Centre Research Institute, Japan; [4]Department of Molecular Pathology, Institute of Biomedical and Health Sciences, Hiroshima University, Japan, Hiroshima, Japan

Introduction: Advanced diffuse type gastric cancer (GC) includes a distinct form, termed scirrhous type GC. It is characterised by highly metastatic and rapid proliferation with abundant stromal fibrosis. Reflecting these characteristics, it carries a poor prognosis compared with other type GC. In this study, we aimed to investigate the role of carcinoma-associated fibroblast (CaF)-derived extracellular vesicles (EVs) on scirrhous type GC progression.
Methods: To check the difference of EVs amount and size between CaFs and normal fibroblasts (NFs), we performed nanoparticle tracking analysis (NTA). To confirm the presence of EVs obtained by ultra-centrifugation, western blot analysis was performed. We also investigate the effect of EV derived from NF and CaF on the invasive activity of HSC-44PE scirrhous type GC cell line by invasion assay. Furthermore, miRNA microarray analysis was performed to identify specific miRNAs in CaF-derived EVs.
Results: NTA showed that the size distribution of both fibroblast-derived EVs were approximately 100–150 nm, and also the EV amounts did not differ between NFs and CaFs. The amount of CD9 and CD63 as EV markers were not significant differences among these fibroblast-derived EVs. We investigated the effect of fibroblast-derived EVs on the invasive activity in HSC-44PE cells. Although total EV amounts and its internalisation did not significantly differ, CaF-derived EVs promoted GC cell invasion, but not NF-derived EVs. These results indicated that the components included in these fibroblast-derived EVs were different. miRNA microarray analysis revealed that oncogenic miRNAs, miR-21 and miR-125b, were highly enriched in CaF-derived EVs as compared with NF-derived EVs.
Conclusion: Our finding indicated that CaF-derived EVs contributed to scirrhous type GC progression by transferring oncogenic miRNAs.

PT10.03

MicroRNA-335-5p is expressed in gastric cancer derived extracellular vesicles and modulates the invasiveness of gastric cancer cells

Iva Polakovicova[1], Lorena Lobos-Gonzáles[2], Nicolás Carrasco[3], Manuel Varas-Godoy[4], Alejandra Sandoval-Bórquez[1] and Alejandro Corvalán[1]

[1]Laboratory of Oncology, Faculty of Medicine, Advanced Centre for Chronic Diseases, Pontificia Universidad Católica de Chile, Santiago, Chile; [2]Fundación Ciencia y Vida, Advanced Centre for Chronic Diseases, Faculty of Medicine, Universidad de Chile, Santiago, Chile; [3]Departament of Chemistry, Faculty of Science, Pontificia Universidad Católica De Valparaíso, Advanced Centre for Chronic Diseases, Pontificia Universidad Católica de Chile, Santiago, Chile; [4]Reproductive Biology Laboratory, Centro de Investigación Biomédica, Faculty of Medicine, Universidad De Los Andes, Bogota, Colombia

Introduction: Gastric cancer (GC) is molecularly complex and ethnically heterogeneous disease. Studies of exclusively Asian origin have reported a controversial role of microRNA-335-5p (miR-335) in GC thus we have analysed the expression of miR-335 in hispanic/Amerindian GC tissues relative to their paired adjacent non-tumour tissues and validated that miR-335 is downregulated in GC. We have also demonstrated that miR-335 overexpression correlates with a variety of biological processes in the tumour cell, including migration, invasion, viability and clonogenic capacities. To further evaluate the role of miR-335, we aimed to investigate the expression of miR-335 in GC derived extracellular vesicles (EVs) and the behaviour of these vesicles on the cell invasiveness.
Methods: EVs were isolated from supernatants from two GC cell lines, a primary tumour-derived cell line AGS and metastatic derived cell line HS746T, from cells transfected with miR-335 mimics, and from plasma patients' samples and characterised by western blot and nanosight.MiR-335 expression levels in cell lines, patients' samples and EVs were analysed by qPCR. First, the invasive properties of both cell lines and cells transfected with miR-335 mimics were studied. Next, the effect of EVs derived from untreated cells and cells transfected with miR-335 mimics on the invasive activity of AGS and HS746T GC cell lines was investigated by invasion assay.
Results: Expression of miR-335 is significantly lower in metastatic HS746T than in primary tumour AGS cell line. AGS also shows less invasive properties. In accordance with these findings cells transfected with miR-335 mimics show significantly decreased invasive properties. MiR-335 is also expressed in the EVs derived from both GC cell lines and patients' plasma. EVs isolated from AGS and HS746T vary in their effect on invasive properties. EVs derived from GC cells overexpressing

miR-335 significantly suppress invasion in both GC cell types though the effect is more pronounced in HS746T cells.
Conclusion: These data complement the clinical relevance of miR-335 and provide further evidence to support the potential role of miR-335 as a metastatic tumour suppressor gene in GC.

PT10.04

High-throughput screening to investigate mechanisms of exosome-driven planar cell polarity signalling

Ainsley Q. Underhill[1,2], Liang Zhang[2], Valbona Luga[3], Mikhail Bashkurov[2], Jacob Belman[2], Mark Jen[2], Jenny Wang[2], Alessandro Datti[2] and Jeffrey Wrana[1,2]

[1]University of Toronto, Toronto, Canada; [2]Lunenfeld-Tanenbaum Research Institute, Toronto, Canada; [3]Cornell University, NY, USA

Non-canonical Wnt signalling is known to regulate planar cell polarity (PCP), an essential process during development. However, aberrant non-canonical Wnt signalling in cancer can also contribute to the dynamics of metastasis and tumour progression. Recent research from my lab identified that breast cancer cells were undergoing increased cellular motility and metastasis by activating the PCP signalling pathways. This pathway was stimulated by extracellular vesicles (EVs) from cancer-associated fibroblasts (CAFs), which were modified by interactions with the breast cancer cells, causing the addition of Wnt11. Other PCP components such as Prickle1, Smurf2, Frizzled6 and Vangl1 were shown to be key to stimulate cellular motility. In my research, I am working to identify additional essential elements up and downstream in this pathway and further investigate this method of PCP signalling. Utilising MATLAB, I have developed a script that can track cells over an imaging series. This system was then tested extensively to ensure it could identify differences in cellular motility, and was observed to produce a dynamic range of ~2 in addition to replicating results produced from manual tracking. With this platform, I can screen for changes in PCP-induced cellular motility, and I recently investigated a cohort of kinase inhibitors, and identify various compounds that affect cellular motility. This included targets from PCP signalling and cytoskeleton control, such as JNK, JAK, FAK, and LIMk. Additionally, other targets included targets from pathways involving protein synthesis, cell proliferation and cycling, metabolomics, and development. I am currently screening a siRNA kinase library available within our facility to further elucidate these results.

PT10.05

Ovarian cancer exosomes have the capacity to mediate the epithelial to mesenchymal transition in target cells

Shayna Sharma[1], Mona Alharbi[1], Katherin Scholz-Romero[1], Carlos Palma[1], Richard Kline[2], Katrina Wade[2], Jacob Estes[2], Andrew Lai[1], John Hooper[3], Gregory Rice[1] and Carlos Salomon[1]

[1]Exosome Biology Laboratory, Centre for Clinical Diagnostics, University of Queensland Centre for Clinical Research, Royal Brisbane and Women's Hospital, The University of Queensland, Brisbane, Australia; [2]Department of Obstetrics and Gynecology, Ochsner Baptist Hospital, New Orleans, LA, USA; [3]Mater Research Institute, University of Queensland, Translational Research Institute, Woolloongabba, Australia

Introduction: Annually, approximately 222,000 women present with ovarian cancer globally. It is considered the most lethal gynaecological cancer. This is often due to the disease being diagnosed late where the 5-year survival rate decreases to approximately 20%. In comparison, the survival rate at an earlier stage is approximately 90%. Therefore, novel diagnostic techniques are being examined. The past decade has seen a great increase in research in the field of extracellular vesicles (EVs), specifically in a subtype of EVs known as exosomes. Therefore, in this study, we explore exosomes in the context of ovarian cancer metastasis.
Methods: Patient derived exosomes were obtained using differential centrifugation and ultrafiltration. Exosomes were characterised using nanoparticle tracking analysis, electron microscopy and western blot. The effect of exosomes on Epithelial to Mesenchymal Transition (EMT) were validated by the ratio of E-cadherin (epithelial marker) to N-cadherin (mesenchymal marker) using western blot and the expression of 84 key genes involved in the EMT (RT^2 Profiler™ PCR Array, QIAGEN) in target cells, CAOV-3 (representative of the primary tumour cells).
Results: Exosomes were identified as spherical vesicles with a typical cup-shape, diameters ranging from 50 to 100 nm, with the expression of TSG101, CD9 and CD81. Expression of WNT5A was increased by 1.16 fold in cells treated with cancer exosomes. Furthermore, cells treated with patient derived exosomes had greater N-cadherin expression although E-cadherin expression was not affected.
Conclusion: Ovarian cancer patient derived exosomes have a role in mediating ovarian cancer progression by influencing the process of EMT.

PT10.06

Exosomes derived from carcinoma-associated fibroblasts induce pre-metastatic niche formation in lung

Jing Kong and Tingjiao Liu

College of Stomatology, Dalian Medical University, Liaoning Sheng, China

Introduction: Salivary gland adenoid cystic carcinoma (ACC) is one of the most common malignant tumours in the oral and maxillofacial region and tends to metastasise to lung. Cancer-associated fibroblasts (CAFs) are a special stromal cell type that actively contributes to tumour growth and malignant behaviour. We explored the function of CAF-derived exosomes in the formation of ACC metastases in mice.
Methods: Exosomes from CAFs were isolated and injected into the tail vein of C57BL-6 J mice. The expression of Fibronection, periostin, and lysyl oxidase (LOX) were examined by immunofluorescent staining. ACC cancer cells were implanted subcutaneously in nude mice and exosomes from CAFs were injected three times a week for 3 weeks. After 4 weeks, the lungs of nude mice were collected and confirmed metastasis by histological examination.
Results: Exosomes from CAFs increased the metastatic behaviour of ACC. CAF-derived exosomes also induced vascular leakiness at pre-metastatic sites. Fibronection, periostin, and LOX are critical for pre-metastatic niche formation.
Conclusion: Our findings demonstrate a critical role for CAF-produced ECM components in premetastatic niche formation and support targeting CAF for the treatment and prevention of metastatic disease.

PT10.07

Exosomal miRNAs derived from mesenchymal phenotype lung cancer cells promote epithelial–mesenchymal transition and serve as potential biomarkers for lung cancer

Yiyao Huang[1], Yue-Ting Tang[2], Si-Hua Qin[1], Yong Xu[3], Taixue An[4], Chun-Chen Liu[1], Qian Wang[1] and Lei Zheng[4]

[1]Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangdong, China; [2]Department of Clinical Laboratory, Zhongnan Hospital, Wuhan University, Wuhan, China; [3]Southern Medical University Affiliated Nanfang Hospital; [4]Department of Laboratory Medicine, Southern Medical University Affiliated Nanfang Hospital, Guangdong, China

Introduction: Epithelial–mesenchymal transition (EMT) is regarded as a critical event during tumour metastasis. Recent studies have revealed changes in and a contribution of proteins in/on exosomes during EMT. microRNA (miRNA) is another important functional component of exosomes. We hypothesised that the miRNA profiles of exosomes may change following EMT and that these exosomal miRNA may promote EMT and metastasis of cancer cells, thus have potential to be the circulating biomakers of lung cancer.
Methods: Transforming growth factor-β (TGF-β1) was used to induce EMT of A549 lung cancer cells. We compared the small RNA profile and function of exosomes from epithelial (E-exosomes) and mesenchymal cancer cells (M-exosomes) by high-throughput sequencing and co-

culture experiments. Then, we preliminarily validated exosomal miRNAs in 2 serum sample sets (25 healthy controls and 22 lung cancer patients) by quantitative real-time RT–PCR. Every research subject signed a prior informed consent that was approved by the Human Research Ethics Committee from Southern Medical University.
Results: The small RNA profile of exosomes was changed following EMT. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that the specific miRNA profile of M-exosomes has the potential to drive signal transduction networks in EMT and cancer progression. Co-culture experiments confirmed that M-exosomes can enter epithelial cells and promote migration, invasion and expression of mesenchymal markers in recipient cells. Exosomal miR-7a, miR-21 and miR-320 expression levels in serum were significantly increased in patients with lung cancer as compared with healthy individuals.
Conclusion: Our research has provided a new insight into the role of exosomes produced by mesenchymal cells, the specifically expressed miRNA in which was associated with the function of EMT and metastasis, and may promote transfer of the malignant phenotype (mesenchymal phenotype) to epithelial recipient cells. These miRNAs differently expressed between healthy individuals and lung cancer patients, and may serve as source of new biomarkers in lung cancer.

PT10.08

Quantitative proteomics of exosome derived from isogenic metastatic and non-metastatic breast cancer in mouse model reveal differential expression of intravasation factors

Jae Won Oh[1], Hye Won Jung[2], Yi Rang Na[2], Seung Hyeok Seok[2] and Kwang Pyo Kim[1]

[1]Department of Applied Chemistry, College of Applied Sciences, Kyung Hee University, Seoul, Republic of Korea; [2]Department of Microbiology and Immunology, Institute of Endemic Disease, Seoul National University College of Medicine, Seoul, Republic Korea

Introduction: Breast cancer is a malignant carcinoma which metastasises anywhere in body but mainly metastasises to bone, lungs, regional lymph nodes, liver and brain. The spread of cancer usually happens through following steps. Firstly, cancer cells invade nearby healthy cells, and the cancer cells penetrate into the circulatory or lymph system, this is called as intravasation.
To investigate physiological roles of exomes in the breast cancer metastasis, it is crucial to reveal the key factor of intravasation from exosomal proteins. Previous studies showed that exosome is significant in metastasis.
Methods: We studied two isogenic breast tumour cell lines, highly metastatic 4T1 and nonmetastatic 67NR, to identify differences in the exosomal proteins. To isolate exosome in *in vivo* environment, 4T1 cells and 67NR cells were injected to BAlB/c mice. Primary cells from the induced tumours by 4T1 and 67NR were isolated and subcultured. The cultured cell media from primary cell cultures were used for exosome isolation by size exclusion chromatography.
We performed quantitative proteomic analysis of prepared exosomes derived from breast cancer in mouse model using isobaric tag based tandem mass tag (TMT) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).
Results: We identified 1254 exosomal proteins and significantly 53 up-regulated proteins and 93 significantly down-regulated proteins in exosomes from 4T1 ($p < 0.05$). Interestingly, migration related pathways and factors are specifically up regulated in exosomes from 4T1. These results suggest that migration factors from exosomes play critical roles in intravasation through specific migration pathways.
Conclusion: Taken together, our exosomal proteome analyses showed key factors of intravasation were enriched in exosomes isolated from metastatic breast cancer.

PT10.09

Tumour exosome-mediated promotion of adhesion to mesothelial cells in gastric cancer cells

Tomohiro Arita, Daisuke Ichikawa, Hirotaka Konishi, Katsutoshi Shoda, Shuhei Komatsu, Atsushi Shiozaki, Daiki Matsubara, Shinpei Ogino, Yuji Fujita, Toshiyuki Kosuga, Hitoshi Fujiwara, Kazuma Okamoto and Eigo Otsuji

Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan

Introduction: Peritoneal metastasis consists of a highly complex series of steps, and the details of the underlying molecular mechanism remain largely unclear. In this study, the effects of tumour-derived exosomes (TEX) on the progression of gastric cancers were investigated in peritoneal metastasis.
Methods: TEX were extracted from cell-conditioned medium by ultracentrifugation. The effects of TEX on the malignant potential of gastric cancer were investigated in adhesion, invasion, and proliferation assays. PCR array as well as western blotting were performed to determine the underlying molecular mechanisms. The molecular changes in mesothelial cell after internalisation of TEX derived from malignant pleural effusion were also con rmed.
Results: TEX were internalised in both mesothelial and gastric cancer cells in a cellular origin non-speci c manner. Internalisation of TEX into mesothelial cells promoted signi cant adhesion between mesothelial and gastric cancer cells, and TEX internalisation into gastric cancer cells signi cantly promoted migratory ability, while internalisation of mesothelial cell-derived exosomes did not. Expression of adhesion- related molecules, such as bronectin 1 (FN1) and laminin gamma 1 (LAMC1), were increased in mesothelial cells after internalisation of TEX from gastric cancer cell line and malignant pleural effusion.
Conclusion: TEX may play a critical role in the development of peritoneal metastasis of gastric cancer, which may be partially due to inducing increased expression of adhesion molecules in mesothelial cells.

PT10.10

Tumour microenvironment affects the composition of endothelial cell-derived extracellular vesicles: impact in tumour progression

Makon-Sébastien Njock[1], Christina O'Grady[2], Franck Dequiedt[2] and Ingrid Struman[1]

[1]Laboratory of Molecular Angiogenesis, GIGA Centre, University of Liège, Belgium; [2]Laboratory of Protein Signalling and Interactions, GIGA Centre, University of Liège, Belgium

The tumour microenvironment plays a crucial role in the progression of tumour growth and metastasis by deregulating various physiological processes including angiogenesis and inflammation. Several studies have previously demonstrated that tumour-derived extracellular vesicles (EVs) are actively involved in the mediation of tumorigenesis by "reprogramming" target cells (e.g. endothelial cells (ECs)) through transfer of pro-angiogenic microRNAs. But the function of EVs released by target cells is poorly studied. Consequently, we sought to determine the composition of EVs released by ECs under tumour microenvironment, and to assess whether these vesicles present different functional properties. Using RNA-seq approaches, we demonstrated that EVs released by ECs in tumour microenvironment context present a specific repertory of microRNAs associated to tumour angiogenesis and inflammatory pathways. Interestingly, some of the dysregulated microRNAs are differently expressed at the cellular and exosomal levels. Furthermore, we showed that these vesicles were able to deregulate angiogenesis pathway by transferring several dysregulated microRNAs to target cells. Currently, we are identifying the molecular targets and pathways modulated by EC-derived EVs under tumour microenvironment.

PT10.11

A role of exosomal miR-10a in bone marrow stromal cells obtained from patients with multiple myeloma

Tomohiro Umezu[1], Satoshi Satoshi[2], Seiichiro Yoshizawa[1], Kazuma Ohyashiki[1] and Junko H. Ohyashiki[2]

[1]Department of Haematology, Tokyo Medical University, Tokyo, Japan; [2]Institute of Medical Science, Tokyo Medical University, Tokyo, Japan

Introduction: Multiple myeloma (MM) is refractory haematologic malignancy. Bone marrow stromal cells (BMSCs) interact with MM cells in the bone marrow (BM), and also create a permissive microenvironment for MM cell growth and survival. Recent evidence indicated that exosome-mediated MM cell-BMSC communication plays an important role in the MM microenvironment. In this study, we investigated the biological property of the exosomes and exosomal miRNAs derived from BMSCs, aiming to establish the emerging strategies to target MM microenvironment to prevent tumour growth and spread.
Methods: BM samples were obtained from MM patients, and BMSCs (mmBMSCs) were isolated using the classical plastic adhesion method. BMSCs from healthy donors (normalBMSCs) were purchased from Lonza Inc. The exosomes were isolated from conditioned medium of BMSCs using Exoquick-TC Reagent (System Biosciences). Cellular and exosomal miRNA profiling was done using a TaqMan low-density array (Applied Biosystems). For functional analysis, the miRNA mimic (Ambion) was overexpressed in BMSCs, and WST-8 (Dojindo) and Caspase-Glo assays (Promega) were performed to determine the impact on cell proliferation and apoptosis, respectively.
Results: We found that exosomal miRNA expression was different between mmBMSCs and normalBMSCs. We found that miR-10a was significantly upregulated in the exosomes derived mmBMSCs, while the expression of miR-10a was low in mmBMSCs. We hypothesised that low expression of cellular miR-10a might be important for survival of mmBMSCs, therefore the miR-10a packaged into exosomes may be released into the extracellular space. Of note is that overexpression of miR-10a inhibited proliferation, and promoted apoptosis in mmBMSCs.
Conclusion: Our results provide the possibility that the inhibition of exosome release may induce mmBMSC apoptosis.

Poster Session PT11 – EVs and the Immune System
Chairs: TBD and Susanne van der Grein 5:15–6:30 p.m.

PT11.01

In vivo analysis of the potential of exosomes isolated from menstrual blood-derived mesenchymal stem cells in regeneration of insulin-producing cells in diabetic type 1 animal model

Elahe Mahdipour, Zahra Salmasi and Nona Sabeti

Department of Medical Biotechnology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Introduction: Diabetes type 1 is characterised by the lack of insulin production as a result of degeneration of insulin-producing beta cells in the pancreas. The autoimmune response against beta cells is the main reason for this disease; therefore, any strategies that help immune response regulation can be beneficial. Studies have shown the effectiveness of mesenchymal stem cells (MSCs) in regulation of T cell response and pancreatic islet repair. However, application of MSCs accompanies the cell therapy safety issue. The unknown fate of injected stem cells is one of the major safety concerns regarding stem cell therapies; therefore, in this study we have used the exosomal secretome of MSCs to regenerate insulin-producing cells.
Methods: MSCs were isolated from menstrual blood as a rich and non-invasive source of MSCs. Exosomes were isolated and characterised using western blot and AFM, TEM techniques. Exosomes were injected intravenously at different time points after induction of diabetes using STZ. Blood glucose and insulin levels were measured at pre-determined time points and animals were sacrificed at day 60 and regeneration of beta cells and insulin production at pancreas were analysed using immunohistochemistry.
Results: flow cytometric and differentiation assays confirmed the characters of MSCs derived from menstrual blood. The presence of CD81, CD63, Tsg-101, Calnexin markers on exosomes was confirmed using western blotting and AFM and TEM analysis verified the presence of purified exosomes. Altogether, the blood levels of glucose and insulin and the histochemistry analyses represented the regenerative potential of exosomes isolated from menstrual blood-derived mesenchymal stem cells in the restoration of insulin-producing cells.
Conclusion: although very successful in preclinical studies, mesenchymal stem cells have still very limited therapeutic applications in clinics mainly because of its safety concerns. Secreted exosome from these cells exerts most beneficial properties of stem cells; however, they follow fewer safety issues as they are not active agents as cells are. This work represents the effectiveness of mesenchymal stem cell-derived exosomes in the regeneration of pancreatic beta cells.

PT11.02

The CD24 receptor induces changes to the surface protein composition of B cell microvesicles with variable effects on RNA and protein cargo

D. Craig Ayre[1], Ian C. Chute[2], Andrew Joy[2], David Barnett[2], Andrew Hogan[1], Marc P. Gruell[3], Lourdes Pena-Castillo[4], Andrew S. Lang[3], Stephen M. Lewis[5] and Sherri L. Christian[1]

[1]Department of Biochemistry, Memorial University of Newfoundland, Newfoundland, Canada; [2]Atlantic Cancer Research Institute, New Brunswick, Canada; [3]Department of Biology, Memorial University of Newfoundland, Newfoundland, Canada; [4]Departments of Computer Science and Biology, Memorial University of Newfoundland, Newfoundland, Canada; [5]Department of Chemistry and Biochemistry, Université de Moncton, New Brunswick, Canada

Introduction: CD24 is a cell surface receptor that promotes the apoptosis of developing B lymphocytes (B cells). We recently found that antibody stimulation of CD24 induces B cells to release CD24-bearing, plasma membrane-derived microvesicles (MVs). As these MVs have not previously been characterised we performed a systematic characterisation of B cell MVs from WEHI-231 B lymphoma cells.
Methods: We examined CD24-induced changes to MV using TEM and nanoparticle tracking. After isolation with the Vn96 peptide, we analysed MV RNA content by RNA-Seq and the MV proteome by nanoLC-MS/MS and western blotting. We analysed the surface receptor repertoire by flow cytometry using bead-based isolation of CD24-bearing MVs.
Results: We found that B cells release MVs of approximately 120 nm, regardless of stimulation, but CD24 stimulation caused an increase in phosphatidylserine-positive CD24-bearing MVs. The RNA cargo from MVs released by both control and CD24-stimulated cells contained predominantly 5S rRNA, but 18S and 28S rRNA were not detected. CD24 stimulation caused a decrease in the abundance of protein coding transcripts and a potential increase in miRNA transcripts, but no statistically significant differential packaging of individual transcripts was detected. The MV proteome was enriched with mitochondrial and metabolism-regulating proteins, and proteins involved in RNA or miRNA shuttling after CD24 stimulation. However, these changes were variable and could not be fully validated by western blotting. Finally, we found that CD24-bearing MVs carry the cell surface proteins Siglec-2 (CD22), CD63, IgM, and, unexpectedly, Ter-119, but do not carry Siglec-G or MHC-II. In response to CD24 stimulation we found that there was a decrease in CD63 and IgM on the surface of MVs, which was not mirrored by changes in cell surface expression.
Conclusion: Overall, our data show that CD24 promotes differentially incorporation of surface receptors during MV biogenesis. While a definitive function for these MVs remains unknown, their composition suggests that they may be involved in release of mitochondrial components from B cells in response to pro-apoptotic stress, with the changes to the surface receptors potentially altering the cell type(s) that interact with the MVs.

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PT11.03

Mesenchymal stem/stromal cell-derived extracellular vesicles attenuate immune responses in two murine models of autoimmune diseases: type 1 diabetes and uveoretinitis

Taeko Shigemoto-Kuroda[1], Joo Youn Oh[2], Dong-Ki Kim[1], Hyun Jeong Jeong[2], Se Yeon Park[2], Hyun Ju Lee[3], Tae Wan Kim[4], Darwin J. Prockop[1] and Ryang Hwa Lee[1]

[1]Institute of Regenerative Medicine, College of Medicine, Texas A&M University, College Station, TX, USA; [2]Department of Ophthalmology, Seoul National University Hospital, Seoul, Republic of Korea; [3]Laboratory of Ocular Regenerative Medicine and Immunology, Biomedical Research Institute, Seoul National University Hospital, Seoul, Republic of Korea; [4]Department of Ophthalmology, Seoul National University Boramae Medical Centre, Seoul, Republic of Korea

Accumulating evidence shows that extracellular vesicles (EVs) produced by mesenchymal stem/stromal cells (MSCs) exert their therapeutic effects in several disease models. We previously demonstrated that MSCs suppress autoimmunity in models of type 1 diabetes (T1D) and experimental autoimmune uveoretinitis (EAU). Therefore, we herein investigated the therapeutic potential of MSC-derived EVs using our established mouse models for autoimmune diseases affecting the pancreas and eye: T1D and EAU. The data demonstrate that MSC-derived EVs effectively prevent the onset of disease in both T1D and EAU. In addition, the mixed lymphocyte reaction assay with MSC-derived EVs indicated that EVs suppress development of T helper 1 (Th1) cells by inhibiting activation of antigen presenting cells. These results raise the possibility that MSC-derived EVs may be a novel alternative to cell therapy for autoimmune diseases.

PT11.04

Exosomes derived from human autologous conditioned serum are nanocarriers for IL-6 and TNF-alfa

Jamal Ghanam, Shaun Gaji, Mustafa Haddouti, Stephan Irsen, Julio Reinecke, Peter Wehling and Maria Weisshaar

University of Applied Sciences Bonn-Rhein-Sieg, Sankt Augustin, Germany

Introduction: Local injection of autologous conditioned serum (ACS) is a common therapeutic regimen for rheumatoid and orthopaedic diseases (ODs). ACS is obtained by incubating of patients' blood and subsequent centrifugation. During blood incubation, immune cells produce high amounts of growth factors and cytokines, including interleukin-1 receptor antagonist (IL-1ra), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α) and transforming growth factor beta 1 (TGF-β1). The aim of this study was to analyse exosomes release into ACS and their cytokine cargo.
Methods: 8 mL of healthy donor whole blood was left at 37°C for 3, 6, 9 and 24 h in a specialised CE marked medical device to obtain ACS. Polyethylene Glycol (PEG) precipitation method was used to isolate exosomes from ACS. The characteristics of exosomes were determined using transmission electron microscopy (TEM). The exosomes' protein pattern was determined by SDS-PAGE and western blot. ELISA was used to quantify IL-6 and TNF-α carried by isolated exosomes.
Results: SDS-PAGE analysis shows the presence of some intense bands with molecular weight in the range of 25 and 58 kDa, corresponding to the main markers of exosomes, CD9 and CD63 (CD81). Bands intensity increases with incubation time of ACS. TEM analysis shows that the sample obtained after 6 h of incubation contains the highest amount of exosomes (8.77×10^7 exosomes/mL) with an average size between 25 and 58 nm. The western blot shows that all purified exosomes contain CD63 and HSP70 markers. An intense band of CD63 was obtained after 6 h, suggesting exosomes accumulation in ACS. The concentration of IL-6 increases over time to a value of 105.20 ± 13.75 pg mL^{-1} after 24 h. The exosomal content of TNF-α decreases in time from a value of 92.14 pg mL^{-1} (t_0) to 0.40 pg mL^{-1} (t_{24}).
Conclusion: Incubation time affects exosomes release and protein inventory. Results from SDS-PAGE, western blot and TEM reveal the presence of exosomes isolated by PEG precipitation. These nanoparticles derived from a biological treatment for ODs carry players in inflammatory reaction (IL-6 and TNF-α). More researches including measurement of other cytokines are required to shed light on the involvement of ACS' exosomes in inflammation management of ODs.

PT11.05

IL35-coated exosomes facilitate an expanded impact of regulatory T cells-mediated suppression

Yusuke Tomita[1], Ewa Jankowska-Gan[1], Ying Zhou[1], Dario Vignali[2] and William Burlingham[1]

[1]Department of Surgery, Division of Transplantation, University of Wisconsin-Madison, WI, USA; [2]Department of Immunology, University of Pittsburgh, PA, USA

Introduction: We reported that donor-specific splenocytes transfusion (DST) plus anti-CD40L costimulatory blockade treatment causes allo-specific regulation approximately 5 weeks after the tolerisation. The regulation may include IL10, TGFb and IL35 (both Ebi3 and p35 subunit) secreted by allo-specific regulatory T (Treg) cells. We observed exosomes-like structures on the surface of both $Foxp3^+$ and Foxp3 negative CD4 T cells.
Hypothesis: (1) $Foxp3^+$ Treg cells are the main source of surface Ebi3 (sEbi3) acquired by bystander CD4 T cells after tolerisation. (2) IL35 will be secreted as components of exosomes by antigen-specific Treg cells.
Methods: CBA ($H2^k$) spleen cells were injected i.v. on day 0 into a two types of double reporter transgenic mice (C57BL/6, $H2^b$ background): (1) ones which expressed YFP under the Foxp3 and TdTomRed under the Ebi3 promoter [$Ebi3^+$ mice], and (2) ones in which both reporters were present, but Ebi3 production was knocked out [$Ebi3^{Floxed}$ mice]. Anti-CD40L blockade (MR-1) was injected i.p. into the mice of 125 ug dose on day 0, 2 and 4. Mice were sacrificed on day 35, spleens were harvested, restimulated with allo-specific CBA antigens overnight, and purified exosomes by ultra-centrifugation. In order to investigate functions of IL35 containing exosome purified from tolerised mice, we used ELISA, trans vivo-delayed type hypersensitivity linked-suppression assay and heart transplantation.
Results: By ImageStream population microscopy, the sEbi3 appeared to be secreted as exosomes by the Treg cells and captured by bystander CD4 non Treg cells. ELISA was able to provide exosome detection, and CD81 enriched exosomes could be captured in ELISA by CD39-, CD73- or Ebi3-specific, but not by p35- or p28-specific coating antibodies. However, both p35- and Ebi3-specific antibodies could significantly ($p < 0.05$) reverse suppression caused by adding the exosomes to a TT-specific recall response, indicating that both chains of IL35 were present and active in suppression. Finally, CBA-tolerised donor strain (B6) exosomes administered day. 0 substantially prolonged graft survival of a B6 to CBA heart transplant (mean survival times, 22 days, $p < 0.05$ vs. untreated groups).
Conclusion: IL35-coated exosomes facilitate an expanded impact of allo-specific Treg cells in peripheral tolerance.

PT11.06

Soluble factors, not extracellular vesicles, are key determinants of MSC: T cell suppression

Anastasia Cheng[1], Natalia de França Shimabukuro[2] and Inés Colmegna[1]

[1]McGill University, Montreal, Canada; [2]Research Institute of the McGill University Health Centre, Montreal, Canada

Introduction: Mesenchymal stromal cells (MSCs) possess potent immune modulatory properties and are promising candidates for the treatment of chronic inflammatory diseases. It is not clear whether MSC derived extracellular vesicles (EV) recapitulate MSC suppressive effects on T cell proliferation and thus could be potential alternatives to cellular therapy.
Methods: Human adipose tissue-derived MSCs ($n = 7$) were characterised according to the minimal criteria proposed by the International Society for Cellular Therapy. 72-hour conditioned media (CM) was collected from resting, and cytokine primed (IFN-γ + TNF-α) MSC. Exosomes were purified from CM by ultracentrifugation and characterised by flow cytometry, nanoparticle tracking analysis (NTA), and transmission electron microscopy. EV depletion was performed by filtration of CM with 100 kDa MWCO and confirmed by NTA. Suppression of proliferating T cells by either (1) MSC (contact dependent vs independent conditions), (2) MSC CM, (3) EV-Free CM, or (4) MSC exosomes (EXO) was assessed in 4-day allogeneic co-culture systems.
Results: MSC remain potent suppressors of T cell proliferation in the absence of direct cell contact, emphasising the relevance of soluble factors and possibly the role of EV (n = 6, contact 86.4 ± 10.4 vs transwell 87.9 ± 11.0, % T cell inhibition, $p > 0.05$). MSC priming increased EV release ($n = 7$, resting 3.4 ± 1.9 × 10^9 vs primed 9.8 ±4.9 × 10^9 EVs/ml, $p = 0.02$), and T cell inhibition by MSC CM ($n = 7$, resting CM 27.7 ± 8.0 vs. primed CM 33.6 ± 5.8, % T cell inhibition, $p = 0.02$). However, fractionation of MSC CM showed that EV were not responsible for T cell inhibition ($n = 7$, CM 35.5 ± 11.5 vs. EV-free CM 31.3 ± 13.5, % T cell inhibition, $p > 0.05$). Moreover, enrichment of MSC EXO (size: 100 nm, markers: CD90/CD81/CD63) did not impact immunopotency ($n = 7$, EXO 10.9 ± 5.8 vs. CM 10.1 ± 6.0, % T cell inhibition $p > 0.05$).
Conclusion: Non-EV soluble factors (<100 kDa) of the MSC CM are mainly responsible for the MSC:T cell suppression.

PT11.07

The role of apoptotic cell disassembly in immunogenic cell death and antigen presentation

Sarah Caruso, Rochelle Tixeira, Thanh Kha Phan, Sara Oveissi, Mark Hulett and Ivan Poon

La Trobe Institue for Molecular Science, Melbourne, Australia

Introduction: Disassembly of apoptotic cells into extracellular vesicles called apoptotic bodies, once considered a random fragmentation event, has recently been shown to consist of highly regulated morphological steps. It has been suggested that apoptotic bodies may aid efficient clearance by phagocytic cells and potentially carry antigen, ultimately promoting immunity towards dying cells. In cancer therapy this offers the potential to develop an anti-tumour immune response. Therefore, this study aims to determine the molecular factors that regulate cell disassembly and examine functional role of apoptotic bodies in eliciting anti-tumour immunity.

Methods: Squamous cell carcinoma and lymphoma cells were induced to undergo anti-Fas or UV-mediated apoptosis *in vitro*. Simultaneously, the key regulators of apoptotic cell disassembly, rho-kinase 1 (ROCK1) and pannexin 1 (PANX1) channel, were targeted pharmacologically and cell morphology and apoptotic body formation was monitored by confocal microscopy and flow cytometry, respectively. To determine the role of apoptotic bodies in immunogenicity, assays assessing clearance and antigen presentation were used.
Results: Targeting ROCK1 and PANX1 during cancer cell apoptosis inhibited and enhanced apoptotic body formation, respectively, demonstrating that apoptotic cell disassembly can be manipulated by pharmacological means. Engulfment assays demonstrated that cells undergoing enhanced disassembly are cleared more effectively by dendritic cells. These data suggest that cell disassembly can promote cell clearance by antigen presenting cells.
Conclusion: Overall, this study demonstrated that apoptotic cell disassembly can be manipulated by targeting key regulators. Enhanced apoptotic body formation by cancer cells can contribute to more effective clearance by dendritic cells and potentially aid antigen presentation. This has implications for cancer therapy, where modulating cell disassembly may be a feasible future approach to generating anti-tumour immunity.

PT11.08

Fine particulate matter (PM2.5) exposure consequences on macrophages polarisation and released extracellular vesicles (EVs)

Amélie Héliot[1], Gauthier Trémolet[1], Yann Landkocz[1], Dorothée Dewaele[2], Frédéric Ledoux[1], Dominique Courcot[1] and Perrine J. Martin[1]

[1]Université du Littoral Côte d'Opale, Dunkerque, France; [2]Centre Commun de Mesures

The increasing incidence of lung diseases in morbidity and mortality rates worldwide generally results from inhalation of air pollution, infectious agents, and various toxic antigens with concomitant immune responses. Airway injury from exposure to particulate matter (PM) is a major risk factor in the development of various lung diseases, including lung cancer (Class I human carcinogen, IARC 2013). Fine PM (diameter below 2.5 µm, PM2.5) may deposit in the alveoli leading to a strong inflammatory reaction by stimulation of the release of different types of mediators from resident or infiltrating immune cells. Among them, macrophages respond to external stimuli with rapid changes in expression of many genes, resulting in the activation of several macrophage phenotypes that played specific regulatory roles in lung cancer processes. Generally, macrophages can be polarised in two distinct phenotypes: the classically activated macrophages (M1), mainly implicated in the pro-inflammatory response, and alternatively activated macrophages (M2), that generally display anti-inflammatory function. They also produce and secrete several factors, among which soluble circulating molecules and extracellular vesicles (EVs) that have a role in orchestrating the inflammatory responses induced by exposure to air pollutants. Depending on their stimulation, macrophages polarised as M1 or M2 can impact their microenvironment to be more or less tumorous. Here, we wondered if PM2.5 exposure has effects on polarisation of human macrophages as well as on profile of the EVs released in response. To answer this question, we have exposed monocytes/macrophages to two concentrations of PM2.5 during 6, 24 and 48 h and assessed their polarisation by evaluating substantial shifts in gene expression (mRNA and miRNA), cytokines production and surface markers. In parallel, EVs have been isolated from exposed macrophages supernatants. Obtained EVs have been characterised for their amount and size distribution by nanoparticle tracking analysis and electron microscopy and miRNA profile has been performed. For the first time we showed that depending on the amount of PM2.5 and time exposure, macrophages display specific phenotype leading to the release of specific EVs.

PT11.09

Identifying exosome binding and internalisation in blood cell subsets by multispectral imaging flow cytometry

Haley R. Pugsley, Sherree L. Friend, Brian E. Hall, Christine E. Probst and Philip J. Morrissey

Amnis part of MilliporeSigma

Introduction: Only recently has the importance of extracellular vesicles as key mediators of intercellular communication been appreciated. Extracellular vesicles are membrane derived structures that include exosomes, microvesicles and apoptotic bodies. Quantifying and characterising exosomes in a reproducible and reliable manner has been difficult due to their small size (50–100 nm in diameter). Exosomes analysis can be done using high-magnification microscopy, however, this technique has a very low throughput. Attempts to analyse exosomes using traditional flow cytometers has been hampered by the limit of detection of such small particles and low refractive index. To overcome these limitations we have employed multispectral imaging flow cytometry that has the advantage of combining high throughput flow cytometry with higher sensitivity to small particles and the added benefit of imaging that can provide visual confirmation of particle integrity and characterisation.
Methods: In this study we use multispectral imaging flow cytometry to investigate the interaction of exosomes with white blood cells. Exosomes derived from Jurkat cells were labelled with anti-human CD63-AF647 and added to human white blood cells. The cells were labelled for immunophenotyping, fixed, and then labelled with anti-human CD63-PE to identify external exosomes.
Results: Plotting internalisation vs. bright detail similarity facilitated the identity three populations: internal exosomes, external/internal exosomes, and external exosomes. Neutrophils, monocytes and lymphocytes were identified by immunophenotyping, the % of each blood cell subset associated with the CD63-AF647 labelled exosomes and whether the exosomes were internalised or external was investigated.
Conclusion: The monocytes had the highest % of cell associated with CD63-AF647 labelled exosomes. And in all of the cell types the majority of the cells associated with CD63-AF647 labelled exosomes were either internalised or partially internalised.

PT11.10

Chimerism-related allotolerance is induce by extracellular vesicle acquisition and reprogramming of host dendritic cells

Diego Lema[1], William Bracamonte-Baran[2], Ewa Jankowska-Gan[1], Frans Claas[3], Jon van Rood[3], Arend Mulder[3] and William Burlingham[1]

[1]Department of Surgery, Division of Transplantation, University of Wisconsin-Madison, WI, USA; [2]Department of Pathology Johns Hopkins University School of Medicine, MD, USA; [3]Department of Immunohaematology and Blood Transfusion, Leiden University Medical Centre, Leiden, The Netherlands

Introduction: Maternal microchimerism (MMc) has been associated with allo-specific graft tolerance in mice and humans. This phenomenon is associated with membrane allo-antigen acquisition (mAAQ, "cross-dressing"), which is mediated by extracellular vesicles (EVs) released by rare maternal resident cells within the offspring. In murine models, MMc-derived EVs induce functional changes in host dendritic cells (DCs), leading to MHC II-restricted allo-peptide presentation (indirect pathway) in co-localisation with mAAQ-induced PD-L1 as an inhibitor signal, thus inducing anergy in indirect antigen-specific effector T cell clones.
Methods: Circulating myeloid DCs (from fresh PBMCs) from a kidney transplant patient (A2,24 B35,57 DR4,11) with excellent renal function 11 years after a 1-HLA haplotype-mismatched sibling transplant (A2,24 B44,57 DR4,11) were stained for HLA-B44, PD-L1 and CD80/86. PBMCs from an HLA-identical non-transplanted sibling were cultured with EVs isolated from plasma of the transplanted patient or a proper syngeneic control and stained likewise. Microscopy flow cytometry (ImageStream) was used to determine mAAQ/co-signalling molecule co-localisation.
Results: In the transplant patient's circulating mDCs a similar pattern of mAAQ as seen in murine models was observed. In cultures patient-derived, but not control-derived, exosomes replicated HLA-B44 mAAQ in myeloid (m)DCs and plasmacytoid (p)DCs. We propose that EVs can induce PD-L1 and CD80/86 expression on human DCs. Furthermore, the co-localisation pattern of PD-L1 or CD80/86 with mAAQ patches in mDCs and pDCs (as determined by microscopy flow cytometry, Image Stream) might modulate the anergy/activation balance of allo-specific T cell clones.

Conclusion: Taken together, these data suggest a potential mechanism similar to that of MMc-induced tolerance in mice by which graft cells are able to induce allo-tolerance via EVs by generating clustering of indirectly presented allo-peptides/MHC complexes with co-inhibitory signals in specific microdomains on recipient mAAQ+ DCs. These findings could explain important mechanisms of chimerism-related tolerance, translate into better transplant outcomes and lead to potential therapeutic targets.

PT11.11

Suppression of inflammatory markers and exosome formation in human lung epithelial cells by near-infrared photobiomodulation

Adam Bartos[1]; Elisa Ghelfi[2]; Magda Bortoni-Rodriguez[1]; Yohann Grondin[1]; James Carroll[3]; Rick Rogers[4]; Rosalinda Sepulveda[1]

[1]Harvard Chan School of Public Health, University of Harvard, Boston, USA; [2]Harvard Chan School of Public Health, Department of Environmental Health, MIPS Program, Harvard University, Boston, USA; [3]THOR Photomedicine Ltd; [4]Harvard T.H Chan School of Public Health, Harvard University, Boston, USA

Exosomes are small secreted membrane vesicles found in tissues, synovial fluids and all other compartments in the body. Intercellular messengers, cargo delivery of effector or signalling macromolecules, and cytokine communication between specific cells are a few of the essential functions ascribed to exosome to date. Exosomes have been reported in a number of acute inflammatory processes in disease or by environmental causes. In this report we investigate the exosome formation and cargo content during inflammation in lipopolysaccharide stressed human A549 human lung epithelial cells and the restoration effect of inflammation and oxidative stress by near-infrared photobiomodulation, as a new therapeutic approach in pulmonary inflammation model.

PT11.12

Tetraspanin CD63 in exosomes derived from human monocytes participates as co-stimulatory molecule in the immunological synapse during dengue virus infection

Pedro Pablo Martínez Rojas, Verónica Monroy-Martínez and Blanca Ruiz-Ordaz

Biomedical Research Institute, National Autonomous University of Mexico, Mexico City, Mexico

Introduction: Dengue fever (DF) is the most important arthropod-borne viral disease in tropical areas. Dengue virus (DENV) infection affects more than 100 million people worldwide each year. DF is caused by any of the four serotypes of DENV and presents a broad clinical spectrum, ranging from a benign self-limiting infection to the severe dengue (SD). DENV mainly infects antigen presenting cells (APC) and monocytes (MO). The contact area between APC and T cells (TC) is called immunological synapse (IS). Tetraspanins are integrins that function as co-stimulatory molecules during TC activation. Tetraspanin CD63 is abundantly in endosomes of APC and MO. The role of tetraspanin CD63 in exosomes, in the absence of CD80/CD86, on the increased TC activity observed in DF is still not described and could be important for the cytokine storm reported in SD cases.
Methods: DENV-2 amplification in C6/36 cells and viral titration by lytic plate assay. Cell culture of THP-1 ATCC TIB-202 (MO) and Jurkat ATCC TIB-152 (TC). Infection of MO with DENV-2 (MOI 1). Exosome isolation by differential ultracentrifugation and continuous density gradient. Exosome morphological characterisation by transmission electron microscopy (TEM), quantification by nanoparticle tracking analysis, and CD63+ identification by flow cytometry. Immunological synapse between TC and CD63+ exosomes by immunofluorescence. Kinetics of mRNA expression of Th1 transcription factors (T-bet, STAT-1, and STAT-4) by RT-PCR and IL-2/IFN-γ quantification by ELISA.
Results: DENV-2 had a titer of 1.0×10^7PFU/mL. Infection caused morphological changes in MO such as filipodia formation and surface adhesions. Exosome concentration was 7.0×10^{10} particles/mL in 2.0×10^7 infected MO. A significant difference ($p < 0.05$) was obtained in concentration between infected and mock MO. All fractions of the gradient were enriched with CD63+ exosomes. TEM showed an exosome heterogeneous (shape and size) population. The rest of the experimental strategy is under development.
Results: In this study, we are evaluating the role of tetraspanin CD63 in exosomes as co-stimulatory molecule in an *in vitro* model of DENV infection.

PT11.13

Uncovering the immunomodulatory potential of mesenchymal stromal cells-derived extracellular vesicles

Raquel Cunha[1], Alisa Ugodnikov[1], Thomas Kuncewicz[1], Helena Lan[1], Heidi Kuang[1], Kelvin Ng[1], Oren Levy[1,2], Rachelle Prantil-Baun[2], Cláudia Lobato da Silva[3], Joaquim Cabral[3], Jeffrey Karp[1] and Donald Ingber[2]

[1]Brigham and Women's Hospital, Harvard Medical School, MA, USA; [2]Wyss Institute for Biologically Inspired Engineering at Harvard University, MA, USA; [3]Department of Bioengineering and iBB-Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal

Introduction: Mesenchymal stromal cells (MSCs) are a promising cell source for cell-based therapies as they exhibit a potent immunomodulatory action in different diseases. It has been reported that MSC secretome is responsible for their immunomodulatory potential. Specifically, MSC-derived extracellular vesicles (EVs) were shown to play a key role in mediating the immunomodulatory effect of MSC. Clinical translation of MSC-EV therapies requires optimised protocols for isolation, characterisation and functional evaluation. This work aims to develop functional assays to assess the immunomodulatory potential of MSC-EV isolated from different MSC donors.
Methods: EVs were harvested from bone marrow MSC and EV isolation was performed by series of ultracentrifugation. EVs were visualised by cryo-electron microscopy, size distribution and concentration were evaluated by nanoparticle tracking analysis and purity by MicroBCA. For the monocyte potency assay, EVs from different MSC donors were incubated with lipopolysaccharide-treated THP-1 cells and secreted inflammation-related cytokines were analysed. For the endothelial potency assay, TNF-α-treated HUVECs (to induce inflammatory stress) were co-incubated with MSC-derived EVs. The secretion of inflammatory cytokines and expression of surface markers were assessed.
Results: Our EV characterisation analysis indicates consistent EV isolation and purity from different MSC donors. The monocyte and endothelial cell-based assays developed were able to distinguish between different MSC-EV donors based on their immunomodulatory properties.
Conclusion: These functional assays are useful tools that can be used to select potent MSC-EV donors towards the evaluation of their therapeutic potential in *in-vitro* and *in vivo* disease models.

Satellite Event
Meet the National and International Societies
Room: Metropolitan Ballroom West and Centre 6:30–8:00 p.m.

Scientific Program ISEV2017
Friday, May 19, 2017

Meet the Expert Morning Session:
Room: Metropolitan Ballroom West and Centre
Session I: EV-Mediated Functional Delivery of Protein Nucleic Acids
Speakers: *Janusz Rak and Raghu Kalluri*
Moderator: *Lucia Languino* 7:45–8:45 a.m.

Room: Metropolitan Ballroom East
Session II: EV Lipids and Lipidomics
Speakers: *Hang Hubert Yin and Alicia Llorente*
Moderator: *Yong Song Gho* 7:45–8:45 a.m.

Room: Harbour Ballroom
Session III: Rigor and Reproducibility in EV Analyses
Speakers: *An Hendrix and Andreas Moller*
Moderator: *Chris Gardiner* 7:45–8:45 a.m.

Oral Sessions
Room: Metropolitan Ballroom West and Centre
Symposium Session 10 – Novel Developments in EV Isolation
Chair: Alain Brisson and Dylan Burger 9:00–10:00 a.m.

OF10.01

Study of exosome therapeutic and diagnostic roles via microfluidic on-demand analysis and harvesting

Mei He[1] and Yong Zeng[2]

[1]Kansas State University, Terry C. Johnson Cancer Research Center, KS, USA; [2]University of Kansas, KS, USA

The finding of exosomes opens new opportunities for liquid biopsy of cancers, and developing nature, non-toxic therapeutic delivery systems. Exosomes play important biological roles via transferring selectively enriched proteins, RNAs, and mitochondrial DNA, which presents distinctive opportunities for liquid biopsy analysis of cancers. Meanwhile, the nano-sized exosomes are highly biocompatible with intrinsic payload and exhibit much stronger antigen loading flexibility, compared to other polymer nano-platforms. In spite of the significant roles in therapeutics and diagnostics, the study and development of the utility of exosomes is hampered by substantial technical difficulties in obtaining sufficient and pure immunogenic exosomes. Current production protocols (e.g. ultracentrifugation and filtration) are un-scalable, often labour-intensive and time-intensive, and in low-yield and purity (<25%). In this work, we report a versatile, scalable microfluidic approach for processing exosomes with precise control and specificity, and on-demand harvesting. Current microfluidic exosome isolation approaches either handle limited quality of exosomes in microliter scale, or the processed exosomes are bound to solid surface/particles and unable to stay intact. We report continuous-flow, light-triggered on-demand harvesting of exosomes over millilitre scale of volumes. We also demonstrated simultaneously multiplexed detection of three tumour markers (EpCAM, CA-125, CD24) for non-invasive diagnosis of ovarian cancer, and monitoring of exosomal surface IGF-1R associated with its intravesicular phosphorylation levels in non-small-cell lung cancer patients plasma. A training set plasma from ovarian cancer patients was obtained from University of Kansas Cancer Center Biospecimen Repository. The blood exosome-based non-invasive diagnosis of ovarian cancer showed significant accuracy and diagnostic power (a.u.c. = 1.0, p = 0.001) compared with the standard Bradford assay (a.u.c. = 1.0, p = 0.0009). We foresee that microfluidic technology will provide game changer roles for exploring the utility of exosomes in therapeutics and diagnostics.

OF10.02

Sequential size exclusion chromatography and density gradient separation of human circulating extracellular vesicles from lipoproteins

Nasibeh Karimi[1], Aleksander Cvjetkovic[1], Su Chul Jang[1], Rossella Crescitelli[1], Rienk Nieuwland[2], Jan Lötvall[1] and Cecilia Lässer[1]

[1]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden; [2]Clinical Chemistry Department, Academisch Medisch Centrum

Introduction: Isolation of extracellular vesicles (EVs) from plasma and serum is of great importance in the field of using EVs as biomarkers for diseases such as cancer. However, blood is one of the most cumbersome body fluids to isolate EVs from, due to the high concentrations of proteins and lipoproteins. The aim of this study was to develop a method to isolate EVs from blood with minimal contamination of lipoproteins.
Methods: Blood was collected from overnight fasting subjects, from which plasma and serum were prepared according to standard protocols. EVs were isolated either by size exclusion chromatography (SEC, 28 fractions) or by different combinations of SEC and iodixonol gradients and cushions. Purity and yield of EVs were determined by electron microscopy (EM), Western blot, nanoparticle tracking analysis and mass spectrometry (LC-MS/MS).
Results: Using SEC, most particles were present in fractions 8–12 as determined by Zeta View®, while the bulk of the proteins were present in fractions 11–28. Vesicle markers, such as flotillin-1 (detected by western blot), and CD9, CD63 and CD81 (detected by ELISA) peaked in fractions 8–10, but were also observed in later fractions. However, EM, LC-MS/MS and western blot (against Apo-A) also showed presence of lipoproteins in fractions 8–12 as well as the later fractions. Lipoprotein particles outnumbered EVs by several fold differences as determined by Zeta View®. When iodixonol density cushion was combined with SEC, the presence of lipoproteins in the EV-enriched fractions was reduced significantly, as determined by EM, Western blot (against Apo-A) and LC-MS/MS.
Conclusion: SEC alone was unable to separate lipoproteins and EVs, whereas an iodixonol cushion followed by SEC improved separation of EVs from lipoproteins.

OF10.03

Isolating neuronal exosomes using cell-type specific protein markers

Emma J. K. Kowal[1], Dmitry Ter-Ovanesyan[2], Michael Burgess[3], Hasmik Keshishian[3], Steven Carr[3], Aviv Regev[1,3] and George M. Church[4]

[1]Massachusetts Institute of Technology, MA, USA; [2]Harvard University, MA, USA; [3]Broad Institute, MA, USA; [4]Harvard Medical School, MA, USA

Introduction: As all cell types secrete exosomes, human biofluids contain a mixture of vesicles from different cell types. Exosomes have tremendous potential as a new class of diagnostics, but their utility is hampered by the the difficulty of determining which exosomes come from which cells.
Methods: We used a combination of methods to identify proteins that are specific to neuron exosomes. We differentiated human induced pluripotent cells (iPSCs) into neurons and then collected exosomes from these neurons. We performed mass spectrometry to identify neuron exosome markers and then developed a computational pipeline to determine which exosome markers are specific to neurons. We then optimised a protocol to efficiently isolate exosomes bearing these markers from heterogenous mixtures of vesicles.
Results: We have found hundreds of proteins present in neuron exosomes, but most of these proteins are not neuron specific. We have identified transmembrane proteins that are neuron specific by overlapping our results with other gene expression and human proteomics datasets. We have further developed a pulldown protocol to isolate neuron specific exosomes from human biofluids.
Conclusion: We have developed an approach for determining cell-type specific exosome protein markers, and demonstrate a proof of principle with neuron exosomes. We have also developed an exosome isolation method which uses these markers to extract neuron-specific exosomes from human biofluids such as cerebrospinal fluid (CSF). We envision this method will be useful in diagnosing a variety of neurodegenerative diseases.

OF10.04

Liquid biopsy on a chip: isolation of exosomes and detection of surface biomarkers for early diagnosis of cancer

Navneet Dogra[1,2], Carlos Cordon-Cardo[2], Jungreem Woo[2] and Gustavo Stolovitzky[1,2]

[1]IBM; [2]Icahn School of Medicine, NY, USA

Introduction: Exosomes are an exciting target for "liquid biopsies". However, isolation of exosomes and detection of their surface biomarkers remains an ongoing challenge. We have developed a nanoscale DLD (Deterministic lateral displacement) device that brings capabilities with size based sorting of colloidal particles at the tens of nanometres scale. Furthermore, we have successfully demonstrated on-chip separation of exosomes and detection of important surface biomarker on exosomes derived from cancer cells.
Methods: Nanofluidic pillar array is manufactured in an SiO_2 mask using optical contact lithography, electron beam (e-beam) and deep ultra violet lithography. Exosomes are derived from prostate cancer cell lines and prostate cancer patients.
Results: We demonstrate size-based separation and quantification of exosomes. Combined with fluorescence microscopy, our technology can sort and identify multiple epitopes simultaneously on single exosomes surface.
Conclusion: These extremely exciting preliminary results indicates the potential of this technology for sorting exosomes and detection of certain disease related biomarkers from plasma, urine, serum or circulating tumour-derived exosomes.

Room: Metropolitan Ballroom East
Symposium Session 11 – EVs in Tumour Metastasis
Chair: Lei Zheng and Yves DeClerck 9:00–10:00 a.m.

OF11.01

Oncosomes as a novel liquid biopsy biomarker for quantifying metastatic cancer dynamics in real-time

Florence Deng[1], Yohan Kim[1], Andrew Chun-Him Poon[2], Tom Liao[1], Karla Williams[3] and Hon S. Leong[1]

[1]Western Ontario, Ontario, Canada; [2]University of Western Ontario, Ontario, Canada; [3]University of British Columbia, British Columbia, Canada

Introduction: Tumour cells acquire qualities that enable them to succeed at key steps of the metastatic cascade, but very little is known about how individual cells accomplish these feats in a challenging hemodynamically active environment. Using intravital imaging, we observe that oncosome release is a key event during cancer cell extravasation in various prostate cancer cell lines. Oncosomes are large cell fragments released by cancer cells at various stages of cancer progression. Having observed their release *in vivo* during cancer cell extravasation, we sought to determine at what other stages of metastasis oncosomes were released.
Methods: Using PC-3, LnCAP and Du145 cells, intravenous injection into the chorioallantoic membrane (CAM) of chick embryos, a gold standard of visualising cancer cell extravasation, was employed and confocal resonance scanning microscopy was used to visualise the release of oncosomes and other smaller extracellular vesicles *in vivo*. Blood at various timepoints was also collected to enumerate the number of CD9+ve and STEAP1+ve oncosomes released by extravasating cells. Primary tumours were also formed and blood collected in the same manner to ascertain the extent of oncosome release *in vivo*.
Results: At the key step of extravasation, arrested cancer cells release oncosomes into the microcirculation which are observed to exhibit a diameter >900 nm and expressing surface antigens found on the surrogate prostate cancer cell such as CD9 and STEAP1. We explored the abundance and biophysical characteristics (size diameter range) of extracellular vesicles (EVs) released during the metastatic cascade and found that oncosomes are not consistently released by primary tumours or metastases and that these large cancer cell fragments are specifically released by actively extravasating cancer cells.
Conclusions: We show that oncosome biogenesis is a specific byproduct of extravasating cells and not by primary tumours or metastatic deposits even in the presence of pro-apoptotic or pro-necroptotic stimuli. Our findings in plasma samples from patients on first-line treatment for metastatic prostate cancer support the concept of oncosomes as a promising biomarker for monitoring cancer metastasis dynamics in realtime.

OF11.02

Malignant extracellular vesicles carrying MMP1 mRNA facilitate peritoneal dissemination in ovarian cancer

Akira Yokoi[1], Yusuke Yoshioka[2], Yusuke Yamamoto[2], Tomoyasu Kato[3], Hiroaki Kajiyama[4], Fumitaka Kikkawa[4] and Takahiro Ochiya[2]

[1]National Cancer Centre Research Institute, Tokyo, Japan; [2]Division of Molecular and Cellular Medicine, National Cancer Centre Research Institute, Tokyo, Japan; [3]National Cancer Centre Hospital, Tokyo, Japan; [4]Nagoya University Graduate School of Medicine, Nagoya, Japan

Introduction: Advanced ovarian cancers are highly metastatic due to frequent peritoneal dissemination, resulting in a dismal prognosis. However the underlying molecular mechanisms remains unknown. Here, we report for the first time evidences that ovarian cancer-derived extracellular vesicles (EVs) are emerging as important mediators of peritoneal dissemination.
Methods: The mouse model of peritoneal metastasis orthotopically established by injecting 4 types of ovarian cancer cell lines into left ovarian bursa, and EVs was injected intraperitoneally to confirm the metastatic effects. To clarify the detail function of EVs, 2 types of mesothelial cells, which are main components of peritoneum, were used. The EVs derived from cell culture supernatant and patients' ascites were isolated using standard serial ultracentrifugation methods.
Results: Each ovarian cancer cell line possessed different metastatic traits *in vivo*, and the EVs from highly metastatic cells, ES-2 cells, strongly induced metastatic behaviour. Notably, the metastatic cancer EVs efficiently induced apoptotic cell death in mesothelial cells *in vitro* and *in vivo*, resulting in the destruction of the peritoneal mesothelium barrier, and promoted dissemination of cancer cells in peritoneal cavity. Whole transcriptome analysis showed that MMP1 was significantly elevated in mesothelial cells treated with ES-2 EVs, and intact MMP1 mRNAs were selectively packaged in the EVs. Importantly, MMP1 expression in ovarian cancer is tightly correlated with a poor prognosis, particularly in stage I patients. Moreover, we found MMP1 mRNA-carrying EVs in the ascites of cancer patients, and these EVs also induced apoptosis in mesothelial cells.
Conclusion: Our findings clarify a previously unknown mechanism of peritoneal dissemination via EVs, which can be novel biomarkers of prognosis, and suggest a new therapeutic strategy for inhibiting metastasis by disrupting the EVs.

OF11.03

Cancer stem cell exosomal tetraspanins network regulate pancreatic cancer metastasis

Shuo Liu, Jun Li, Teng Wang and Shijing Yue

School of Medicine Nankai University, Nankai, China

Introduction: Exosomes derive from multiple cell types and are found in all body fluids. Many studies indicate that exosomes represent the most important, including long distance intercellular communication system. Tumour exosomes play a pivotal role on cancer metastases. Tetraspanins are the transmembrane 4 superfamily (TM4SF) proteins, which enrich in exosomes and involve in a multitude of functional activities. However, it is obscure that the components of cancer stem cells (CSCs) derived exosomes and the functional role of tetraspanins network in the progress of cancer metastases.
Methods: In this study, we used sphere culture and FACS sorting to obtain the specific CSCs population. Ultracentrifugation was used to prepare and enrich cancer cell and CSCs derived exosomes. The component and tetraspanins network of CSCs derived exosomes were analysed by MALDI-TOF for proteome and RNA-seq for mRNA and miRNA. The pancreatic cancer cell lines Aspc1, CFPAC-1, Bxpc3, Capan1 were used as the models. Knockout mouse was established to explore the regulation of Tspan8 in cancer metastasis.
Results/Conclusion: We found that CD9, CD151, and Tspan8 are enriched in different exosomes derived from cancer cell and CSCs. CD151 and Tspan8 promote the uptake of exosomes by endothelial cell and induce angiogenesis. Furthermore, *in vivo* culture SP-Dio18-labelled exosomes derived from CSCs or cancer cells in mice model indicated that CD151 and Tspan8 increase exosomes targeting liver, spleen, mesentery, pancreases and lung. Coculture exosomes with different cancer cells in SCID mice model demonstrated that exosomal CD151 and Tspan8 promoted pancreatic cancer in liver and lung metastasis. Tspan8 deficient mice reduced the B16 cell metastasis significantly. We concluded that exosomal CD151 and Tspan8 targeting different tissues to form the pre-metastatic niche for inducing metastasis.

OF11.04

Comprehensive EV proteomics revealed EV-driven intercellular communications in gastric cancer microenvironment and macroenvironment

Naomi Ohnishi[1], Risa Fujii[1], Kentaro Murakami[2], Hisahiro Matsubara[2] and Koji Ueda[3]

[1]Japanese Foundation for Cancer Research, Tokyo, Japan; [2]Chiba University, Chiba, Japan; [3]Project for Personalised Cancer Medicine, Cancer Precision Medicine Centre, Japanese Foundation for Cancer Research, Tokyo, Japan

Introduction: Extracellular vesicles (EVs) play various roles in mutual communications between cancer cells and extracellular environment. To understand the significance of EV-mediated protein transportation in cancer development or progression, we developed a high-purity EV isolation tool (EV-Second columns) and performed proteome-wide quantitative profiling of serum EVs derived from gastric cancer (GC) patients or healthy donors.

Methods: Serum samples were collected from 58 individuals (healthy donors, n = 10, GC patients, n = 48). Following isolation of EVs by EV-Second columns based on mixed mode of size exclusion and weak hydrophobic interaction, EV proteins were subjected to LC/MS analysis. Protein identification, label-free quantification, and subsequent statistical analysis were performed on Expressionist proteome server platform. Proteins specifically detected in GC-derived EVs were functionally evaluated.

Results: The LC/MS analysis identified 822 EV proteins in which 13 proteins showed significant up-regulation in GC patients' EVs (t-test, $p < 0.05$, fold change >2.0). Among them, frequent overexpression of PN-1 protein in GC cells (80.0% of undifferentiated carcinoma or 59.1% of adenocarcinoma) was confirmed by multiple tissue array analysis (n = 327). Interestingly, incorporation of PN-1^{++} EVs drastically prevented the recipient cells from chemically-induced apoptosis *in vitro*. Further single cell pH reporter assay revealed that PN-1 enzyme inhibited pre-apoptotic intracellular pH change, leading to survival of cancer cells in, for instance, hypoxic conditions.

CagA, a pathogenic factor of *H. pylori*, was also found in serum EVs from GC patients (1). CagA in GC cell-derived EV was efficiently transferred into recipient cells and induced typical morphological change, indicating that *H. pylori* proteins were transported EVs in blood circulation and may be involved in cancer development and also extragastric diseases. Indeed, *H. pylori* infection increases incidence of non-gastrointestinal diseases such as cardiovascular diseases.

Conclusion: These data suggested that cancer-related EVs are served as key mediators controlling both tumour microenvironment and macroenvironment, which could provide novel mechanisms underlying tumour development or progression.

Reference

1. Shimoda A et al., *Sci. Rep*. 2016; 6: 18346.

Room: Harbour Ballroom
Symposium Session 12 – EVs in Viral Infections
Chairs: Marc-Andre Langlois and Caroline Gilbert 9:00–10:00 a.m.

OF12.01

Communication via extracellular vesicles enhances viral infection of a cosmopolitan alga

Daniella Schatz, Shilo Rosenwasser, Sergey Malitsky, Sharon Wolf, Ester Feldmesser and Assaf Vardi

Weizmann Institute of Science, Rehovot, Israel

Massive oceanic algal blooms that cover thousands of square kilometres, often display a synchronised "boom and bust" dynamics, despite being composed of unicellular organisms.
The cosmopolitan alga *Emiliania huxleyi* belongs to the coccolithophores, a class of unicellular phytoplankton that dominates the modern ocean and mediates the oceanic carbon and sulfur cycles. *E. huxleyi* blooms are routinely terminated following infection by a large, specific, lytic virus, the *Emiliania huxleyi* virus (EhV).
Communication between microorganisms in the marine environment has immense ecological impact by mediating trophic-level interactions and thus determining community structure. However, very little is known about modes of cell-cell communication that may coordinate biotic interactions (e.g. between cells or with grazers, bacteria and viruses) that control the fate of these blooms. Despite recent advances in the studies of extracellular vesicles (EVs) and their role in cell-cell communication in metazoans and protists, almost nothing is known about EV production during microbial interactions in the marine environment.
We investigated the signalling role of EVs produced during interactions between the cosmopolitan alga *E. huxleyi* and its specific virus, EhV. Using Nanosight technology we found that EVs are highly produced during viral infection or when bystander cells are exposed to infochemicals derived from infected cells. Lipidomics and transcriptomic analyses of these EVs demonstrated that they have a unique lipid composition that differs from that of their infected host cells, and their cargo is composed of selected small RNAs that are predicted to target sphingolipid metabolism and cell-cycle pathways. *E. huxleyi* cells can uptake vesicles, consequently leading to a faster viral infection dynamic and prolonging EhV half-life in the extracellular milieu. We propose that extracellular vesicles are exploited by viruses to sustain efficient infectivity and propagation across *E. huxleyi* blooms. Since these algal blooms have immense impact on cycling of carbon and sulfur, this novel mode of cell–cell communication may influence the fate of the blooms and, consequently, the composition and flow of nutrients in the microbial food webs in the ocean.

OF12.02

Apoptotic bodies – a novel Trojan horse for influenza A virus

Georgia Atkin-Smith, Erika Duan, Damien Zanker, Stephanie Paone, Sara Ovessi, Mark Hulett, Weisan Chen and Ivan Poon

La Trobe Institute for Molecular Sciences, Melbourne, Australia

Introduction: For many years the fragmentation of an apoptotic cell into apoptotic bodies (ApoBDs), via a process termed apoptotic cell disassembly, was thought to be a random process dependent mainly on plasma membrane blebbing. However, we have recently demonstrated that monocytes generate long, membrane protrusions, which are beaded in morphology and thus coined beaded-apoptopodia. These beaded-apoptopodia undergo a segmentation-like event to release abundance of ApoBDs. As ApoBDs can facilitate intracellular communication through the trafficking of biomolecules (e.g. DNA, RNA and proteins) and monocytes undergo apoptosis during infection, we asked whether monocyte apoptotic cell disassembly played a role in influenza A virus (IAV) infection, in particular whether ApoBDs could traffic virus biomolecules and aid viral propagation.
Methods: To analyse the formation, content and function of ApoBDs generated from IAV infected monocytes, we used a series of flow cytometry-based assays and mouse infection models. Additionally, we recently developed a novel protocol to isolate ApoBDs from cell culture and tissue samples to high purity for specific analysis.
Results: We first demonstrated that IAV can induce apoptosis and apoptotic cell disassembly in THP-1 monocytes *in vitro* and in mouse monocytes *in vivo*. Secondly, data suggests that IAV proteins, genomic material and lethal virions are distributed into beaded-apoptopodia and ApoBDs of infected monocytes. When incubated with viable cells, ApoBDs derived from IAV-infected cells could induce apoptosis and viral infection, whereas control ApoBDs (UV treatment) did not. Strikingly when administered intranasally to mice, ApoBDs from infected THP-1 cells could induce a severe inflammatory response, viral infection and also aid viral antigen presentation.
Conclusion: For the first time, these results demonstrate that apoptotic cell disassembly may act as a double edged sword during infection by both aiding viral propagation and immune detection. As we have recently identified a series of commonly used pharmaceutical compounds which can manipulate the disassembly process, further studies may unveil novel therapeutic strategies to combat viral infection.

OF12.03

Extracellular vesicles released by HIV-infected CD4+ T cells promote the secretion of proinflammatory cytokines by uninfected bystander lymphocytes: role of hypoxia inducible factor 1 alpha

Gabriel Duette[1], Pehuen Pereyra Gerber[1], Andrea Morales[1], Julia Rubione[1], Alvaro Lopez Malicia[1], Maria Pia Holgado[1], Clovis Palmer[2] and Matias Ostrowski[1]

[1]INBIRS Institute, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina; [2]Burnet Institute, Melbourne, Australia

Introduction: Chronic T cell activation and dysfunction are hallmarks of HIV infection. Taking into consideration that T cell metabolism influences T cell functionality, we hypothesised that CD4+ T cell dysfunction during HIV infection could be associated to virus-induced metabolic alterations. A critical transcription factor in the coordination of T cell metabolism, differentiation and effector function is Hypoxia inducible factor-1 alpha (HIF-1). Herein, we analysed the role of extracellular vesicles in the bystander modulation of HIF-1 activity and CD4+ T cell function during HIV infection.
Methods: CD4+ T cells isolated from the blood of healthy donors were infected *in vitro* with HIV mutants unable to produce progeny viral particles. Extracellular vesicles were isolated by differential centrifugation and/or analysed by immunocapture on CD63-coated beads followed by detection with fluorescently-labelled antibodies. The role of EVs released by HIV infected cells in bystander CD4+ T cell metabolism and function was assessed.
Results: HIV-1 infection triggers HIF-1 expression and activity, promoting aerobic glycolysis and the production of the proinflammatory cytokines IL-17A and interferon-gamma. Moreover, HIV-1 induces the HIF-1-mediated secretion of Extracellular Vesicles. These vesicles, in turn, promote HIF-1 activity and the secretion of gamma-interferon in bystander cells.<
Conclusion: HIV infection induces the activity of HIF-1 in productively infected cells and the secretion of EVs that, in turn, induce glycolytic activity and a proinflammtory phenotype in bystander CD4+ T cells. Overall, our results suggest that EVs released by HIV infected cells contribute to chronic immune activation and inflammation in HIV-1-infected patients.

OF12.04

Extracellular vesicles carry HIV Env and facilitate HIV infection of human lymphoid tissue

Anush Arakelyan[1], Wendy Fitzgerald[2], Soina Zicari[2], Christophe Vanpouille[2] and Leonid Margolis[1]

[1]Eunice-Kennedy National Institute of Child Health and Human Development, MD, USA; [2]Section of Intercellular Interactions, Eunice-Kennedy National Institute of Child Health and Human Development, MD, USA

Introduction: Cells productively infected with HIV-1 release virions along with extracellular vesicles (EVs). The biogenesis, size and physical properties of EVs resemble those of viruses, particularly of HIV. Here, we found that EVs carry viral surface proteins and these EVs *ex vivo* affect HIV replication in human lymphoid tissue, where critical events of HIV infection occur *in vivo*.

Methods: We analysed individual EVs in HIV-1 suspensions using our recently developed magnetic nanoparticle (MNP) based technology. We immunocaptured EVs released by HIV-infected PBMCs with 15-nm MNPs coupled to antibodies recognising viral surface protein Env, separated the captured particles on magnetic columns, and analysed them with a flow cytometer. The captured EVs were distinguished from the virions, also captured by these anti-Env MNPs, by the presence of CD45 and/or acetylcholinesterase (AchE), the proteins that are not incorporated in virions.

Results: Flow cytometry analysis of particles immunocaptured from HIV-1-infected PBMC with anti-Env MNPs revealed that 52.6 ± 5.7% (n = 5) and 40 ± 0.6% (n = 3) of them were CD45+ or AchE+, respectively, thus identified as EVs. Next, we evaluated the effects of these EVs on HIV infection of human lymphoid tissue *ex vivo*. Depletion of an HIV-1 preparation of CD45+ EVs resulted in a significantly lower level of infection 54.5 ± 8.0% (n = 4, p = 0.03) compared to mock depleted preparation. To evaluate whether this effect was caused by the depletion of general CD45+ EVs or by EVs that carry Env, we first depleted the viral preparation of particles carrying Env with MNPs coupled to anti-Env antibodies (2G12- or PG16) and then additionally depleted with anti-CD45 MNPs. Depletion of the viral preparation of particles (virions and EVs) that are recognised by anti-Env antibodies decreased the tissue infection level to 41.6 ± 6.1% (n = 3, p = 0.03) in the case of PG16 and to 43.8 ± 7.5% (n = 4, p = 0.003) in the case of 2G12. Additional depletion of these preparations of CD45-positive EVs did not result in a significant (p > 0.2) additional decrease of infection.

Conclusions: EVs that carry viral Env facilitate HIV-1 replication and constitute a factor in HIV infection. These EVs may become to be a new target for anti-HIV therapy.

Room: Metropolitan Ballroom West and Centre 10:30–11:45 a.m.

Plenary Session 02 – Plasma Membrane and Cellular Vesicles
Chairs: *Xandra Breakefield, PhD, Alissa Weaver, MD, PhD*
Speakers: Clotilde Thery, PhD (Institut Curie, Paris, France)
Pathways and Mechanisms of Extracellular Vesicle Formation
Juan Bonifacino, PhD (National Institutes of Health, Betheda, MD, United States)
Mechanisms and Functions of Lysosome Positioning

Room: Metropolitan Ballroom West and Centre
Featured Abstracts
Chairs: Xandra Breakefield and Alissa Weaver 11:45–12:30 p.m.

LBO.08

Real-time quantification of multi-vesicular body-plasma membrane fusion reveals modulation of exosome release by G protein-coupled receptor signaling

Frederik J. Verweij[1], Maarten P. Bebelman[2], Juan J. Garcia-Vallejo[3], Marc G. Coppolino[4], S. Rubina Baglio[5], Hans Janssen[6], Jacques Neefjes[7], Matthijs Verhage[8], Jaap M. Middeldorp[5], Anoek Zomer[9], Jacco van Rheenen[9], Jaco Knol[10], Richard de Goeij- de Haas[10], Sander R. Piersma[10], Ilse Hurbain[11], Graça Raposo[12], Martine J. Smit[13], Connie R. Jimenez[14], Ruud F. G. Toonen[8], Guillaume Van Niel[15] and D. Michiel Pegtel[16]

[1]Exosomes Research Group Department of Pathology VU University Medical Center Cancer Center Amsterdam (CCA); [2]Exosomes Research Group, Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands; [3]Dept. Molecular Cell Biology & Immunology, VU University Medical Center, Amsterdam, The Netherlands; [4]Dept. of Molecular and Cellular Biology, University of Guelph, Guelph, ON N1G 2W1, Canada; [5]Dept. Pathology, Cancer Center Amsterdam, VU University Medical Center, Amsterdam, The Netherlands; [6]Electron Microscopy Facility, Netherlands Cancer Institute NKI-AvL, Amsterdam, The Netherlands; [7]Department of Chemical Immunology, Leiden University Medical Center LUMC, Leiden, The Netherlands; [8]Department of Functional Genomics and Department of Clinical Genetics, Center for Neurogenomics and Cognitive Research, VU University Amsterdam and VU Medical Center; [9]Cancer Genomics Netherlands-Hubrecht Institute-KNAW & University Medical Centre Utrecht, Utrecht, The Netherlands; [10]Department of Medical Oncology, Cancer Center Amsterdam, VU University Medical Center, Amsterdam, The Netherlands; [11]Institut Curie, PSL Research University, Paris, France; [12]Centre National de la Recherche Scientifique and Institut Curie, PSL Research University, Paris, France; [13]Division of Medicinal Chemistry, Amsterdam Institute for Molecules Medicines and Systems, VU University Amsterdam, Amsterdam, The Netherlands; [14]Department of Medical Oncology, Cancer Center Amsterdam, VU University Medical Center; [15]Institut Curie, PSL Research University, CNRS, UMR 144, Paris, France /Center for Psychiatry and Neuroscience; [16]Exosomes Research Group, Department of Pathology, VU University Medical Center, Amsterdam, Netherlands

Introduction: Exosomes are endosome-derived small extracellular vesicles (EVs) implicated in cell-cell communication and secreted by Multi-vesicular Bodies (MVBs) fusing with the plasma membrane. Current techniques to study exosome physiology are based on isolation procedures post-secretion, precluding direct dynamic insight into the mechanics of exosome biogenesis and the regulatory mechanisms involved in exosome release. Here we propose real-time visualization of MVB-PM fusion to overcome these limitations.
Methods: We designed tetraspanin-based optical reporters that spot MVB-PM fusions using live Total Internal Reflection Fluorescence (TIRF) microscopy and dynamic Correlative Light-Electron Microscopy (CLEM).
Results: Detailed single-cell analysis demonstrates that MVB-PM fusion activity is reduced by depletion of the tSNAREs SNAP23 and Syntaxin-4 and can be induced by stimulation of the Histamine H1 Receptor (H1HR). Activation of this G protein-coupled receptor in HeLa cells increases Ser110 phosphorylation of SNAP23 promoting MVB-PM fusion.
Summary/Conclusion: Using this single-cell live imaging approach, we highlight the modulatory dynamics of MVB exocytosis that will increase our understanding of exosome physiology and help identify druggable targets in exosome-associated pathologies.
Funding: This work was funded by the Dutch Cancer Fund (KWF-5510) a CCA grant to DMP and an EMBO long-term fellowship to FV (EMBO ALTF 1383-2014).

FFA-01

Analysis of tumour-infiltrating innate immune cells after uptake of glioblastoma-derived extracellular vesicles *in vivo*

Erik R. Abels[1], Sybren Maas[1], Lieke van de Haar[1], Xuan Zhang[1], Shilpa Prabhakar[1], Charles Lai[2], Suzanne Hickman[3], Marike Broekman[1], Xandra O. Breakefield[1] and Joseph El Khoury[3]

[1]Department of Neurology and Radiology and Program in Neuroscience, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA; [2]National Tsing Hua University; [3]Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Boston, MA, USA

Introduction: Extracellular vesicles (EVs) including exosomes are 50–1000 nm vesicles whose contents correspond to that of the donor cell and consists of proteins, RNA and DNA. These vesicles are shed by all cells and have been shown to play a role in various diseases, including glioblastoma (GBM). Previously, we have shown that EVs shed by glioblastoma cells can influence the cells in the tumour (micro)environment, including brain-resident microglia and infiltrating monocytes/macrophages, making them more tumour-supportive. The aim of this study was to study the influence of GBM-derived EVs on tumour-associated microglia, monocytes and macrophages *in vivo*.
Methods: To this aim, we labeledmurine GL261 glioma cells using the palmitoyated-GFP reporter, which labels membranes including those of EVs. In addition, membrane-bound *Gaussia* luciferase (GlucB) was used as a bioluminescence reporter. The resulting syngeneic GL261.palmGFP. GlucB cells were implanted intracranially in adult C57/BL6.CCR2-RFP$^{\pm}$ mice. The CCR2 promoter drives RFP expression in monocytes and cell types derived from them. Four weeks after implantation, brains were harvested and prepared for flow cytometric cell sorting. mRNA expression in the different cellular clusters was subsequently analysed using Illumina mRNA sequencing.
Results: In tumour-bearing brains, EV-associated palmGFP uptake could be detected and subsequently sorted in the different subpopulations of microglia. In microglia from tumour-bearing mice, we detected the activation of both immune-stimulatory and tumour-supportive pathways. In detail, we show microglia that took tumour derived EVs (including exosomes) have a reduced ability to sense danger signals in their environment and have upregulated Stat3, interleukin 4, interleukin 10 and angiogenesis pathways.
Conclusion: In conclusion, our data show that brain resident microglia take up EVs on site and that EV uptake is associated with a stronger response of both immune-stimulatory and tumour-supportive pathways *in vivo*.

FFA-02

Impaired angiogenesis and cancer metastasis by exosomes in Tspan8 deficient mice

Jun Li, Shuo Liu, Teng Wang and Shijing Yue

School of Medicine, Nankai University, Nankai, China

Introduction: Tetraspanins are highly conserved 4-transmembrane proteins which form molecular clusters with a large variety of transmembrane and cytosolic proteins. By these associations tetraspanins are engaged in a multitude of biological processes, which become further expanded by the location of tetraspanin complexes in specialised membrane microdomains, called tetraspanin-enriched microdomains (TEM). TEM provide a signalling platform and are poised for invagination and vesicle formation. These vesicles can be released as exosomes, indicating that tetraspanins are also engaged in cell contact-independent intercellular communication. The tetraspanin Tspan8 is a cancer initiating cell

marker in gastrointestinal tumours promoting migration and invasion via associated integrins and proteases. Depending on the cellular integrin profile it supports angiogenesis and can induce disseminated intravascular coagulation.
Methods: To shed light on its activities in non-transformed cells, we generated a Tspan8 knockout (ko) mouse, comparing tumour growth, angiogenesis and wound healing with that of control mice, mice with a targeted deletion of CD151 ($CD151^{ko}$) and $Tspan8/CD151^{ko}$ mice. The serum exosomes were collected from wild type, $Tspan8^{ko}$, $CD151^{ko}$, $Tspan8/CD151^{ko}$ mice. *In vitro* and *in vivo* assays were used to explore the function of Tspan8, CD151 and the tetraspanin deficient exosomes.
Conclusion: $Tspan8^{ko}$ and $Tspan8/CD151^{ko}$ mice show no abnormal breeding and develop alike control mice. No changes in organ structures or behavioural anomalities were observed. With the exception of a slightly impaired TH1-mediated DTH responses, hematopoiesis and immune reactivity were not affected. No changes were seen in local growth and metastasis of a wt melanoma, but angiogenesis was slightly reduced. The latter also accounted for a pancreatic adenocarcinoma that additionally, and distinct to $CD151^{kd}$ mice, showed reduced metastatic capacity in Tspan8 and $Tspan8/CD151^{kd}$ mice. Impaired angiogenesis was confirmed *in vitro* in the aortic ring assay. Mehtylcholanthrene-induced tumours in $Tspan8^{ko}$ and $CD151^{ko}$ mice confirmed reduced migratory and angiogenic activity, which prohibited tumour progression most efficiently in $Tspan8^{ko}/CD151^{ko}$ mice. Serum exosomes derived from $Tspan8^{ko}$, $CD151^{ko}$, $Tspan8^{ko}/CD151^{ko}$ mice impaired the migratory activity of endothelial cells. The similar results were also observed during wound healing, where epithelial cell migration, vessel recruitment and matrix reorganisation were severely impaired, most strongly by the serum exosomes derived from $Tspan8/CD151^{ko}$ mice.
Conclusion: We concluded that neither $Tspan8^{ko}$ nor $Tspan8^{ko}/CD151^{ko}$ mice exert a pathological phenotype. However, defects in migration and angiogenesis become obvious *in vivo* and *in vitro* during wound healing and tumour progression.

FFA-03

Presence of glypican-1 on extracellular vesicles fails to discern pancreatic cancer from benign pancreatic diseases

Fabrice Lucien[1], Vivian Lac[2] and Hon S. Leong[3]

[1]Lawson Health Research Institute, Ontario, Canada; [2]OVCARE; [3]Western Institute, Ontario, Canada

Introduction: Pancreatic cancer is the fourth leading cause of cancer-related deaths in North America and the five-year survival rate approximately 5%, with most patients dying within several months. One of the biggest problems faced by physicians is that pancreatic cancer is clinically silent at its early stages and symptoms associated with the disease often only appear once the cancer has invaded neighbouring tissues or has metastasised to distant sitesthus providing little opportunity for therapeutic intervention. Currently, there is no effective early-detection screening test for pancreatic cancer exists because current biomarkers suffer from poor specificity to pancreatic cancer and are commonly elevated in benign pancreatic diseases (BPD). A recent study suggests that the presence of Glypican-1 (GPC1) on extracellular vesicles (EVs) accurately identifies early- or late-stage pancreatic cancer from healthy individuals or BPD patients (1). Hence, we hypothesise that a liquid biopsy enumerating GPC1-positive EVs will represent a blood test capable of discerning pancreatic cancer from BPD.
Methods: Plasma from patients with BPD, resected pancreatic cancer, and metastatic (stage IV) pancreatic cancer have been analysed for GPC1-positive EVs ranging from 100–1000 nm in diameter using nanoscale flow cytometry. Since GPC1 is expressed in several other types of cancers, we also tested the utility of a test enumerating EVs concurrently positive for GPC1 and glycoprotein-2 (GP2), a pancreas-specific marker.
Results: The majority of pancreatic cancer patients possessed low GPC1 EV counts. Neither GPC1 nor GPC1-GP2 levels are significantly elevated in pancreatic cancer patients compared to patients with BPD. The lack of difference in EV counts between resected and metastatic cancer groups reveals a lack of correlation of GPC1 levels with tumour burden. The sensitivity and specificity of the GPC1 EV test were 26.67% and 87.50%, respectively, whereas the sensitivity and specificity for the GPC1+GP2 EV test were 23.33% and 90.00%, respectively.
Conclusion: The presence of GPC1, solely or in conjunction with GP2 analysis, was unable to effectively distinguish between BPD and pancreatic cancer. Consequently, GPC1 may not be useful in the early detection of pancreatic cancer.

Reference

1. Melo SA et al., *Nature.* 2015; 23: 177–182..

Room: Metropolitan Ballroom West and Centre
Symposium Session 13 – Novel Technologies in EV Characterisation
Chairs: Joanne Lannigan and Rienk Nieuwland 1:30–3:00 p.m.

OF13.01

Extracellular vesicles isolated in evaporating droplets

Hwapyeong Jeong[1], Youseok Hyun[1], Yogesh Gianchandani[2] and Jaesung Park[1]

[1]Pohang University of Science and Technology, Pohang, Republic of Korea; [2]University of Michigan, MI, USA

Introduction: Extracellular vesicles (EVs) commonly contain membrane-associated tetraspanin, CD9, CD63 and CD81. However, no decisive markers specifically distinguish subpopulations of EVs. Instead, subpopulations of EVs are assumed to possess different physical as well as biochemical characteristics due to the different biogenesis. To exploit the physical characteristics of subpopulations of EVs for isolation, various methods, such as differential centrifugation and size exclusion chromatography, has been developed. However, due to multi-physical factors dependence of isolation method, a subpopulation of EVs are not completely distinguishable from other populations. In this study, EVs were isolated spatially based on their size in evaporating droplet. We then find that the size of EVs is correlated with expression levels of certain tetraspanin proteins and confirmed that the possibility of this method can be used for diagnosis.
Methods: EVs from WM 266-4 and MCF-7 were suspended in a droplet that was placed on a glass with various temperature gradient. EVs were stained with anti-CD9, CD63 and CD81. After evaporation, EVs formed ring near the contact line of the droplet. The expression levels of surface proteins on dried ring patterns were observed under a fluorescence microscope. For downstream analysis, EVs form prostate cancer patient (PCa) were collected from evaporating droplet. Expression of PCA-3, and PSMA in the collected EVs from cancer patients were analysed by qPCR and western blotting.
Result: Chromatography using capillary and Marangoni flows provides sufficient chromatographic resolution to spatially separate nanoparticles by size with ~48 nm resolution. Using methods based on the deposition and transfer of microdroplets, subpopulations of EVs were spatially separated based on size. The average size of CD63 high-expressed EVs was larger than CD9- or CD81-high-expressed EVs. For prostate cancer diagnosis, both the level of PCA-3 expression and PSMA expression in the isolated lager EVs from PCa was lower than the starting EV population.
Conclusion: A remarkable consequence with this method is that the size of EVs is correlated with expression levels of certain tetraspanin proteins. Further, the size of EVs from prostate cancer patients is correlated with expression levels of prostate cancer-associated markers.

OF13.02

Single cell analysis revealed cell-to-cell variations in physiologic state influence EV secretion

Valya Ramakrishnan[1], Johnny C. Akers[1], Wei Cai[2], Yi-Huan Tsai[2], Roger Chiu[2], Yu-Hwa Lo[2], Bob S. Carter[1] and Clark C. Chen[1]

[1]Center for Theoretical and Applied Neuro-Oncology, University of California, San Diego, CA, USA; [2]Department of Electrical and Computer Engineering, University of California, San Diego, CA, USA

Introduction: Most studies in the biogenesis of extracellular vesicle (EV) are performed using a population of cells, under the assumption that every cell in the population exhibits comparable biology. Here we perform analysis of EV secretion rate at the single cell level and provide data suggesting heterogeneity in single-cell behaviour in terms of EV secretion.
Methods: Lithographic patterning of polydimethylsiloxane (PDMS) was used to construct a platform supporting culturing of GFP-labelled GBM3 (a short-term passaged, patient-derived glioblastoma line). Cell growth was monitored by time-lapse GFP microscopy. Arrays of single cells in culture are overlaid with glass slides coated with CD63 antibody to capture secreted EVs. Quantitation of EVs captured on this slide was performed by probing with a biotinylated anti-CD9 antibody. Direct quantitation of Qdot fluorescence was performed after the addition of streptavidin conjugated Qdots to biotinylated EVs.
Results: Single cells derived from GBM3 culture that exhibit identical growth rate and cell morphology showed a wide range of EV secretion rates, ranging from 3 to 72 EVs secreted per hour. When fitted to Gaussian models, three distinct model distributions were identified, suggesting distinct cell states. The median secretion rates of this distribution were 10, 18, and 38 EVs per hour. The rate of EV secretion did not significantly vary as a function of the cell cycle. Single-cell qPCR analysis showed that cells in distinct model distributions differed in the expression of OLIG2 and SOX2, two genes that play a critical role in maintaining and defining glioblastoma cancer stem cell state. We showed that single cells spontaneously transition between these cell states.
Conclusion: Single cell analysis suggests that dynamic heterogeneity in cell states influences EV secretion.

OF13.03

Time-resolved surface enhanced raman spectroscopy for characterising extracellular vesicles

Tatu Rojalin[1], Heikki Saari[2], Petter Somersalo[2], Saara Laitinen[3], Tapani Viitala[2], Zachary J. Smith[4] and Marjo Yliperttula[2]

[1]University of Helsinki, Helsinki, Finland; [2]Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland; [3]Finnish Red Cross Blood Service, Helsinki, Finland; [4]Department of Precision Mechanics and Precision Instrumentation, University of Science and Technology of China, Hefei, Anhui

Introduction: The aim of this work is to develop a platform for characterising extracellular vesicles (EVs) by using gold-polymer nanopillar surface-enhanced Raman spectroscopy (SERS) substrates simultaneously circumventing the photoluminescence-related disadvantages of Raman with a time-resolved approach. Currently, straightforward, label-free and fast EV characterisation methods with low sample consumption are warranted. In this study, SERS spectra of red blood cell (RBC) and platelet (PLT) derived EVs were successfully measured and their biochemical contents analysed using multivariate data analysis techniques.
Methods: RBC and PLT vesicles were isolated using differential centrifugation. 2 μLs of EV samples were pipetted on the gold-polymer nanopillar SERS substrates that provided Raman signal amplification. The SERS spectra were recorded with a pulsed picosecond 532 nm laser in combination with a single-photon counting array detector. Complementary EV characterisation was carried out by nanoparticle tracking analysis and western blot.
Results: The acquired SERS spectra were in abundance of distinguishable spectral features and the interfering photoluminescent spectral backgrounds were effectively suppressed. Very small volumes of EV samples were needed. Multivariate data analysis revealed that RBC and PLT vesicles can be accurately identified using this platform. In our previous studies Raman spectra of single RBCs had been recorded using the Raman laser trap system. Herein, comparison between RBC EV SERS and RBC laser trap spectra demonstrated strong resemblance to each other reporting on the biochemical similarities between the RBC EVs and their parent cells. These perceptions supported the feasibility of the designed SERS method in the context of EV characterisation.
Conclusions: The introduced label-free, time-resolved SERS method provides detailed biochemical information on the investigated RBC

and PLT EV samples. SERS measurements of biological samples, such as EVs, typically suffer from photoluminescence backgrounds swamping important SERS spectral features; these difficulties can be overcome by resolving the photoluminescence and SERS signals in the time domain. The developed platform is a promising tool for characterising various types of EVs in general.

OF13.04

Raman spectroscopy for the label-free identification of the source-related biochemical fingerprint of extracellular vesicles

Alice Gualerzi[1], Stefania Niada[2], Marta Gomarasca[2], Silvia Picciolini[3], Valeria Rossella[4], Carlo Morasso[1], Renzo Vanna[1], Marzia Bedoni[5], Fabio Ciceri[6], Maria Ester Bernardo[4], Anna Teresa Brini[2] and Furio Gramatica[1]

[1]Laboratory of Nanomedicine and Clinical Biophotonics LABION, Fondazione Don Gnocchi; [2]IRCCS Galeazzi Orthopaedic Institute, Università degli Studi di Milano; [3]Laboratory of Nanomedicine and Clinical Biophotonics LABION, Fondazione Don Gnocchi – University of Milano-Bicocca; [4]TIGET, Paediatric Immunohematology and Stem Cell Programme, San Raffaele Hospital; [5]Laboratory of Nanomedicine and Clinical Biophotonics LABION, Fondazione Don Carlo Gnocchi ONLUS; [6]Haematology and Bone Marrow Transplantation Unit, San Raffaele Hospital

Introduction: Extracellular vesicles (EVs) are crucial intercellular communication vehicles for bioactive molecules with diagnostic and therapeutic relevance. The recent growth of studies on EV effects in disease pathogenesis, tissue regeneration, and immunomodulation has led to the application of multiple isolation and characterisation techniques poorly standardised and with scarcely comparable outcomes. Current methods for EV characterisation mainly rely on general biomarkers and physical features that do not mirror the actual heterogeneity of vesicles. Raman spectroscopy is a label-free, rapid, non-destructive, sensitive method that can become a useful tool for the biochemical characterisation and discrimination of EVs from multiple cell types.
Methods: Human mesenchymal stromal cells from bone marrow and adipose tissue, and dermal fibroblasts were cultured for 72 h in serum free conditions. Ultracentrifuged vesicles obtained from conditioned media were analysed by confocal Raman microspectroscopy with 532 nm laser sources in the spectral ranges 500–1800 cm^{-1} and 2600–3200 cm^{-1}. Multivariate statistical analysis (PCA-LDA) and classical least squares (CLS) fitting with reference lipid molecules (cholesterol, ceramide, phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and GM1) were performed on recordings obtained on air-dried drops of EV suspensions.
Results: When vesicles were irradiated, Raman bands of nucleic acids, proteins, and lipids (cholesterol, phospholipids) were visible in the spectra providing a biochemical fingerprint of the considered vesicles. CLS fitting allowed the calculation of the relative contribution of lipids to the recorded spectra. By Raman spectroscopy we can clearly distinguish vesicles originated by different cell-types with good accuracy (around 93%) thanks to biochemical features typical of the cell/tissue of origin.
Conclusion: Our results suggest Raman spectroscopy as a valuable approach for EV characterisation prior to their use in complex disease models. In particular, requiring minimal amount of samples and no sample preparation, Raman analysis can be used as a routine quality check method for EVs before *in vitro* or *in vivo* use, being also more informative compared to other complementary techniques.

OF13.05

EV-TRACK: transparent reporting and centralising knowledge in extracellular vesicle research

Jan Van Deun[1], Pieter Mestdagh[2], Patrizia Agostinis[3], Geert Berx[4], Jan Gettemans[5], Bernd Giebel[6], Andrew F. Hill[7], Suresh Mathivanan[8], Esther N.M. Nolte-'t-Hoen[9], Lorraine O'Driscoll[10], Michael Pfaffl[11], Susmita Sahoo[12], Johannes Swinnen[13], Clotilde Théry[14], Guillaume Van Niel[15], Marca H.M. Wauben[9], Kenneth Witwer[16], Olivier De Wever[17], Jo Vandesompele[2] and An Hendrix[17]

[1]Laboratory of Experimental Cancer Research, Cancer Research Institute Ghent (CRIG), Ghent University, Ghent, Belgium; [2]Center for Medical Genetics, Cancer Research Institute Ghent (CRIG), Bioinformatics Institute Ghent (BIG), Ghent University, Ghent, Belgium; [3]Cell Death Research & Therapy (CDRT) Lab, KU Leuven University of Leuven, Leuven, Belgium; [4]Department of Biomedical Molecular Biology, Cancer Research Institute Ghent (CRIG), Ghent University, Molecular and Cellular Oncology Lab, Inflammation Research Centre, VIB, Ghent, Belgium; [5]Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium; [6]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden; [7]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Victoria, Australia; [8]La Trobe Institute for Molecular Science; [9]Department of Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [10]School of Pharmacy and Pharmaceutical Sciences and Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland; [11]Division of Animal Physiology and Immunology, TUM School of Life Sciences Weihenstephan, Technical University Munich, Munich, Germany; [12]Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai, New York, USA; [13]Laboratory of Lipid Metabolism and Cancer, Department of Oncology, LKI – Leuven Cancer Institute, KU Leuven, Leuven, Belgium; [14]Institut Curie, PSL Research University, INSERM U932, Paris, France; [15]Institut Curie, PSL Research University, CNRS, UMR 144, Paris, France; [16]The Johns Hopkins University School of Medicine; [17]Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Cancer Research Institute Ghent (CRIG), Ghent University, Ghent, Belgium

Introduction: Transparent reporting is a prerequisite to facilitate interpretation and replication of extracellular vesicle (EV) experiments. We convened an international consortium to develop a resource to improve the rigour and interpretation of experiments, record the evolution of EV research and create a dialogue with researchers about relevant experimental parameters.
Methods: We analysed 1226 articles with keywords "exosomes" or "extracellular vesicles" published in 2010–2015. Publications that included multiple sample types or isolation methods were separated into multiple entries, resulting in 1742 experiments. Experiments were analysed using a matrix containing 115 parameters related to sample type and EV isolation/characterisation methods. The database is freely accessible and expandable, allowing online deposition of new experiments (http://evtrack.org)
Results: To assess current practice in EV experiments, we performed an in-depth analysis of recorded data in the EV-TRACK knowledgebase. This revealed heterogeneity in EV isolation methods and inconsistent reporting of experimental parameters. Differential ultracentrifugation is the most used isolation method (>50%) but with a large heterogeneity in centrifugation steps. In less than 20% of experiments a density gradient was implemented to obtain or at least validate results. Quality controls are often omitted, with more than 2 proteins being checked in 40% and non-EV enriched proteins in less than 15% of experiments (dependent on sample type). From these analyses, 9 relevant experimental parameters were extracted and condensed into a single metric, the EV-METRIC (to MEasure Transparent Reporting of Isolation and Characterisation methods). It represents a checklist to assess the completeness of reporting of generic and method-specific information necessary to interpret and repeat an experiment. The EV-TRACK platform is a knowledge centre for EV biology and methodology that allows data queries, coaches users by providing EV-METRICs and involves them in decision-making on future improvements to the platform.
Conclusion: Established for and supported by the EV research community, the EV-TRACK platform aims to ensure that experimental guidelines are timely, and transparently met. It is accessible at http://evtrack.org.

OF13.06

Size and concentration determination of extracellular vesicles as small as 50 nm in diameter at a rate beyond 10,000 EV/s

Jean-Luc Fraikin[1], Leonie de Rond[2], Chi Hau[2], Franklin Monzon[1] and Edwin van der Pol[3]

[1]Spectradyne LLC; [2]Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands; [3]Biomedical Engineering & Physics and Vesicles Observation Centre, Academic Medical Centre

Introduction: Clinical applications of extracellular vesicle (EV) characterisation methods demand both fast count rates to detect rare particles (e.g. tumour-derived EV in plasma) and sensitivity spanning the entire EV size range (~50–1000 nm). Traditional methods fail to meet one or both metrics. Here, a rapid and commercially available on-chip technology, microfluidic resistive pulse sensing (MRPS), is validated in a head to head comparison against five established techniques and used to characterise a variety of clinically relevant samples. MRPS is shown to be a rapid and highly sensitive method with significant potential for use in clinical applications.

Methods: MRPS was first validated using two standard samples: a mixture of reference beads and EV from human cell-free urine (n = 5). The samples were analysed by MRPS (Spectradyne, nCS1) and the results were compared to measurements of equivalent samples obtained by nanoparticle tracking analysis (NTA, Nanosight NS-500), tunable resistive pulse sensing (TRPS, iZon qNano), flow cytometry (Apogee A50-Micro) and tunnelling electron microscopy (TEM, Philips CM10). Finally, the utility of MRPS in clinically-relevant applications was evaluated using real-world EV samples: plasma, blood bank concentrates, and two tumour cell lines (LNCaP, PC-3).

Results: MRPS successfully characterised the standards and revealed significant differences between the real-world EV samples. Measured peak diameters in the bead mixture agreed with TEM to within an average of 8%. A power law dependence of EV concentration c, on diameter d, of $c \sim d^{-4.2}$ was observed in the urinary vesicles over five orders of magnitude in concentration (on a size range of 50–1000 nm), with remarkable agreement to TEM and TRPS measurements of similar samples. Measurements of the clinically-relevant EV samples demonstrated an average sample turnaround time under 10 minutes, and revealed other power law distributions and significant, quantitative differences between samples.

Conclusion: MRPS proved a powerful technique for measuring the size and concentration of EV in clinically relevant samples, demonstrating accuracy higher than NTA and similar to TRPS with faster measurement time. The performance and ease-of-use of this technique support its potential for EV-based clinical applications.

Room: Metropolitan Ballroom East
Symposium Session 14 – EVs in Cardiovascular Disorders
Chairs: Chantal Boulanger and Mike Davis 1:30–3:00 p.m.

OF14.01

The pericardial fluid exosomes as new cell-to-cell communicators worsening ischaemic heart disease in diabetes

Jaimy Saif[1], Sezin Aday[1], Giovanni Biglino[1], Kate Heesom[1], Maryam Anwar[2], Gianni Angelini[1], Enrico Petretto[3] and Costanza Emanueli[4]

[1]University of Bristol, Bristol, United Kingdom; [2]Imperial College London, London, United Kingdom; [3]Duke-NUS Medical School, NC, USA; [4]Bristol Heart Institute, University of Bristol, Bristol, United Kingdom

Cardiovascular disease is prevalent in type 2 diabetes mellitus (T2DM) and is associated with both macrovascular disease and microangiopathy, contributing to ischaemic heart disease(IHD). Functional studies focussing on exosomes in human biological fluids are important to investigate the relevance of exosome-based communications in human pathophysiology. The pericardial fluid (PF) located in the pericardial sac, is an ultrafiltrate of plasma obtained by capillary permeability and osmotic pressure from the epicardium and interstitial fluid underlying the myocardium. We previously showed that PF exosomes promote cardiovascular cell survival and angiogenesis *in vitro* and *in vivo*. Here we hypothesise that the vascular protective and reparative actions of PF exosomes are perturbed by T2DM, which could hijack and modify these exosomes to amplify and spread its deleterious programme. Under ethical approval, PF samples were collected (at the beginning of the operation) from T2DM and non-diabetic (NDM) patients with IHD undergoing CABG surgery. The quality of exosome preparations were validated by Nanosight, Western blotting for exosome antigens and by electron microscopy. The internalisation of exosomes by recipient cells was confirmed by confocal microscopy. T2DM exosomes increased apoptosis in recipient ECs in hypoxia and reduced the formation of capillary networks on Matrigel in EC maintained in high glucose in hypoxia. Proteomic profiling of PF exosomes from T2DM(vs NDM) showed significantly higher expression of proteins involved in cardiac hypertrophy, fibrosis and cell death. A higher expression of WD-repeat proteins was seen in T2DM exosomes. WD-repeats regulate APAF-1 (apoptotic peptidase activating factor-1) self-association for apoptosome formation and procaspase activation. Western blot confirmed higher expression of APAF-1 in DM-exosomes. MicroRNA profiling of PF exosomes showed downregulation of pro-angiogenic microRNAs, including let-7b-5p which targets and inhibits capsase-3 expression, in PF exosomes from T2DM. In conclusion, the study shows pro-apoptotic and anti angiogenic effect of PF exosomes from patients with IHD and T2DM. T2DM exosomes also showed altered proteomic and microRNA profile, suggesting increased formation of apoptosomes and delayed angiogenesis.

OF14.02

Endothelial cell-derived extracellular vesicles in acute myocardial infarction

Naveed Akbar[1], Janet Digby[1], Thomas Cahill[1], Abhijeet Tavare[1], Sushant Saluja[1], Sam Dawkins[1], Laurienne Edgar[1], Nadia Rawlings[1], Klemen Ziberna[1], Eileen McNeil[1], Errin Johnson[1], Alaa Aljabali[1], Rebecca Dragovic[1], Mala Rohling[1], Grant Belgard[2], David Greaves[1], Keith Channon[1], Daniel Anthony[1] and Robin Choudhury[1]

[1]University of Oxford, Oxford, United Kingdom; [2]Verge Genomics

Background: The mechanism by which acute myocardial infarction (AMI) mobilises monocytes from the spleen into peripheral blood and induces transcriptional activation remains unknown. Here we report the role of endothelial cell (EC)-derived extracellular vesicles (EVs) in monocyte mobilisation and transcription activation.
Methods and Results: EV were isolated by ultra-centrifugation and analysed by nanoparticle tracking analysis, TEM, western blot, ELISA and qPCR. AMI increased plasma EV in humans ($p < 0.01$) and mice ($p < 0.01$). Plasma EV display EC integrins including VCAM-1, which is enriched after injury ($p < 0.05$). *In vitro*, pro-inflammatory but not anti-inflammatory cytokines increase EC-EV release ($p < 0.001$) and enrich for EV-VCAM-1 ($p < 0.05$). *In vivo* EC-EV localise to the spleen and mobilise splenic-monocytes in naive mice ($p < 0.01$). *In vitro* EC-EV stimulates monocyte motility ($p < 0.05$) and enhance their migration in response to chemokines ($p < 0.05$) in a VCAM-1 dependent-way ($p < 0.05$). 12 miRNAs are enriched in plasma EV following AMI including EC-associated miR-126-3p, which regulates cell motility genes including plexin-b2, a negative regulator of cell motility. EC-EV exposure to monocytes lowers plexin-b2 mRNA ($p < 0.01$) and enhances integrin and chemokine receptor expression ($p < 0.01$).
Conclusion: EC-EV release may be one mechanism by which the injured myocardium signals monocyte mobilisation and transcriptional activation post-AMI.

OF14.03

Hypoxic pre-conditioning on human CD34+ stem cells enhances exosome therapeutics of ischemic tissue repair through ETS-1-regulated pathway

Yaxuan Liang[1], Prabhu Mathiyalagan[1], Sol Misener[2], Douglas Losordo[3] and Susmita Sahoo[1]

[1]Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai, New York, USA; [2]Feinberg Cardiovascular Research Institute, Feinberg School of Medicine, Northwestern University, IL, USA; [3]Caladrius Biosciences, NY, USA

Previous studies in our lab have discovered that therapeutically important human $CD34^+$ hematopoietic stem cells secrete exosomes (Exo) to induce angiogenic activity both *in vitro* and *in vivo*. MicroRNA microarray analysis suggests that CD34+ exosomes (CD34Exo) carry proangiogenic miRNAs, such as miR-126, which affect the therapeutic function of CD34Exo.
Here, we hypothesise that hypoxic treatment of CD34+ stem cells can modulate the miRNA content and regenerative efficacy of CD34Exo. Exosomes from human CD34+ cells cultured under hypoxia (H-Exo) were more proliferative, anti-apoptotic and angiogenic *in vitro*, compared to exosomes from cells under normoxia (N-Exo). In a mouse model of hind limb ischemia (BalbC nude), H-Exo treatment significantly enhanced limb perfusion, increased capillary density, and prevented ischemic limb amputation compared to N-Exo. To identify the factors responsible for improved therapeutic function of H-Exo, we compared both protein and miRNA components of H- and N-Exo. Using 2D-DIGE and mass spectrometry analysis, we found that expression of major proteins in H-Exo did not differ significantly than N-Exo. However, expression of proangiogenic miRNAs was increased significantly in H-Exo (e.g. miR-210 and miR-126) compared to N-Exo. We have examined the role of ETS-1, a transcription factor induced by hypoxia-inducible fator-1 (HIF-1), in regulating the expression of miR-126. We propose that HIF-1/ETS-1 regulatory mechanisms affect the expression of exosomal miR-126 under hypoxia. These results are being confirmed using siRNA silencing and using HIF hydroxylase inhibitor dimethyloxalylglycine.
We conclude that hypoxia-induced miR-126 expression in CD34 cell-derived exosomes stimulating exosomes-mediated angiogenesis and therapeutic recovery via ETS-transcriptional pathway. Our work has important clinical implications to improve therapeutic angiogenesis, especially in diabetic and cardiovascular patients, who have stem cells with diminished angiogenic potential.

OF14.04

Circulating exosomes correlate with metabolic syndrome severity and evoke changes of mitochondrial dynamic which are associated with endothelial dysfunction

Marine Malloci[1], Madlyne Esnault[2], Zainab Safiedeen[2], Severine Dubois[3], Jerome Boursier[4], Frederic Gagnadoux[4], Ramaroson Andriantsitohaina[1], Gilles Simard[3] and M. Carmen Martinez[1]

[1]INSERM U1063; [2]INSERM UMR1063 – University of Angers, France; [3]INSERM U1063/Angers University Hospital, Angers, France; [4]CHU d'Angers, Angers, France

Metabolic syndrome (MetS) is characterised by a cluster of interrelated risk factors -hyperglycemia, dyslipidemia, hypertension and obesity- leading to an increased risk of cardiovascular events. Exosomes can be considered as new biomarkers of different pathologies, and can be involved in intercellular communication. Here, we hypothesise that exosomes could be implicated in MetS-associated endothelial dysfunction. Therefore, circulating exosomes of non-MetS subjects and MetS patients have been isolated from plasma and characterised. Thereafter, exosomes effects on endothelial function were analysed by measuring nitric oxide (NO) and reactive oxygen species (ROS) production and mitochondrial dynamic proteins, on human endothelial aortic cells (HAoECs). Whereas circulating levels of exosomes positively correlated with the number of MetS criteria, their size was negatively correlated with the number of MetS criteria. Moreover, exosomes were mainly originated from leukocytes and platelets in both non-MetS and MetS subjects. In HAoECs, exosomes from MetS patients decreased NO production through the inhibition of the endothelial NO-synthase activity. Furthermore, exosomes from MetS patients increased Mitosox-associated fluorescence, reflecting enhanced mitochondrial ROS production, leading to increased protein tyrosine nitration. This was associated with a decreased expression of mitochondrial fusion proteins (Mfn1 and OPA1) and an increase of FIS1 expression, without modification of mitophagy. Furthermore, MetS exosome treatment decreased mtDNA/nDNA ratio but had no effect on expression of mitochondrial biogenesis actors (PGC1, NRF1 and TFAM). These results provide evidence that exosomes from MetS patients could be new biomarkers for this pathology and may contribute to endothelial dysfunction in MetS, by decreasing NO production, increasing oxidative stress and disturbing mitochondrial dynamic. Thus, exosomes may be a future target to prevent and treat this pathology.

OF14.05

Fibronectin regulates exosome secretion by human vascular smooth muscle cells

Catherine M. Shanahan[1], Chris Molenaar[1], D. Michiel Pegtel[2], Frederik Verweij[3], Maddy Parson[4] and Alexander N. Kapustin[1]

[1]King's College London British Heart Foundation Centre of Excellence, London, United Kingdom; [2]Exosomes Research Group, Department of Pathology, VU University Medical Centre; [3]Exosomes Research Group Department of Pathology VU University Medical Centre, Cancer Centre Amsterdam (CCA), Amsterdam, The Netherlands; [4]Kings College London, United Kingdom

Introduction: Atherosclerotic plaque rupture causes stroke and heart attack. Plaque stability is defined by the composition of the extracellular matrix and the balance between vascular smooth muscle cells (VSMCs) and macrophages in the fibrous cap. We recently identified fibronectin as a novel marker of plaque vulnerability. Moreover, ablation of circulating fibronectin prevented VSMC accumulation in the fibrous cap indicating that fibronectin may regulate VSMC proliferation and migration.
Methods: Exosomes were isolated using differential ultracentrifugation. To visualise MVBs and exosome secretion, VSMC were transfected with CD63-GFP, vinculin-RFP or CD63-pHluorin using electroporation and analysed by total internal reflection fluorescence microscopy or spinning disc confocal microscopy (Nikon).
Results: Fibronectin has been identified as a crucial exosomal component regulating tumour cell migration so we studied fibronectin loading into VSMC exosomes. Exogenously added fibronectin-Alexa555 was integrated in the matrix fibrils and endocytosed by VSMC. Internalised fibronectin colocalised with early and late endosome markers and was further secreted in exosomes. Inhibition of exosome secretion using an inhibitor of sphingomyelin phosphodiesterase 3 reduced VSMC migration. Notably, immobilised fibronectin stimulated exosome secretion and inhibition of Arp2/3 blocked this effect. Time-lapse microscopy revealed actin "tails" pushing CD63-positive endosomal organelles indicating that the branched actin network may play a crucial role in the delivery of MVB to exosome exocytosis sites. Using a CD63-pHluorin vector we identified that exosomes are secreted juxtaposed to focal adhesion sites.
Conclusions: In conclusion, fibronectin stimulates exosome secretion by VSMC which in turn, modulates VSMC migration. Modulation of the branched actin network and/or exosome secretion opens a new avenue for atherosclerosis treatment and prevention.

OF14.06

The role of exosomes in mesenchymal stem cell mediated enhancement of cardiac contractility

Joshua Mayourian, Delaine Ceholski, Irene Turnbull and Kevin Costa

Icahn School of Medicine at Mount Sinai, NY, USA

Introduction: An emerging therapy for non-ischemic cardiomyopathy involves the delivery of human mesenchymal stem cells (hMSCs). Clinical trials document modest benefits on cardiac contractility, underscoring a need to better understand and exploit the underlying mechanisms governing hMSC-cardiomyocyte interactome. Recent studies on hMSC-mediated heart therapies demonstrated that paracrine signalling via secreted factors is a crucial mediator of reduced cardiac fibrosis and enhanced angiogenesis. Moreover, hMSC paracrine factors have been shown to impact contractility by altering cardiomyocyte ion channel/pump activity. However, these findings fail to identify the key components of the hMSC secretome, such as exosomes, for enhancing contractility.
Methods: We utilise three-dimensional human engineered cardiac tissues (hECTs) as an *in vitro* model to investigate the role of hMSC exosomes in the enhancement of cardiac contractility. Following baseline hECT contractile function testing on day 5, hECTs cultured in serum-free defined media (SFDM) were replaced with the following treatments: (1) SFDM (Control); (2) hMSC conditioned media (hMSC CdM); (3) SFDM supplemented with hMSC exosomes (hMSC exo); or (4) hMSC exosome-depleted conditioned media (hMSC exo-depl). hECTs were cultured an additional 5 days, and then developed force (DF) was measured again.
Results: The hMSC CdM and hMSC exo treatments led to statistically significant increases in DF, whereas the control and hMSC exo-depl groups were unchanged relative to pre-treatment. hECTs were then snap-frozen for prospective real time quantitative polymerase chain reaction of cardiac-specific, apoptosis, and calcium handling genes, where mRNA levels of SERCA2a and LTCC significantly increased only for hECTs treated with hMSC CdM and hMSC exo, while they significantly decreased for the BAX/BCL2 ratio, an established apoptosis marker.
Conclusion: These findings demonstrate at functional and molecular levels that hMSC exosomes play a key role in hMSC paracrine mediated enhancement of hECT function, motivating further investigation of the key exosomal cargo responsible for these cardioactive effects.

Room: Harbour Ballroom
Symposium Session 15 – EV RNAs as Cancer Biomarkers
Chairs: Andrew Hill and Kendall Jensen 1:30–3:00 p.m.

OF15.01

miR-145 in urinary extracellular vesicles as biomarkers for prostate cancer

Yong Xu[1], Si-Hua Qin[2], Taixue An[3], Yue-Ting Tang[4], Yiyao Huang[2] and Lei Zheng[3]

[1]Southern Medical University affiliated Nanfang Hospital, Guangdong, China; [2]Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangdong, China; [3]Department of Laboratory Medicine, Southern Medical University affiliated Nanfang Hospital, Guangdong, China; [4]Department of Clinical Laboratory, Zhongnan Hospital, Wuhan University, Hubei, China

Introduction: Extracellular vesicles (EVs) are known can be detected in body fluids, and miRNAs in EVs may serve as disease biomarkers. Hydrostatic filtration dialysis (HFD) is a method separating EVs without the need for trained laboratory personnel and heavy initial investment. Increasing evidence suggests circulating miRNAs in serum and urine may be potential non-invasive biomarkers for prostate cancer (PCa). In the present study, we aimed to investigate the whether HFD is suitable for urinary EVs isolation and weather such reported miRNAs can be detected in urinary and serum EVs as PCa biomarkers.
Methods: We compared the efficiency of HFD and conventional ultracentrifugation (UC) in isolating urinary EVs. Subsequently, EVs were isolated from the urine of patients with PCa, patients with benign prostate hyperplasia (BPH) and healthy individuals. Differential expression of 5 PCa-related miRNAs were measured in urine and paired serum EVs using SYBR Green-based quantitative reverse transcription-polymerase chain reaction.
Results: The performance of HFD was similar to UC except lower EVs concentration. In miRNA yield, both HFD and UC meet the needs of follow-up analysis. 4 miRNAs, which were reported abundant in human urinary EVs, were found no significant differences in HFD-EVs and UC-EVs. We validated miRNAs in 60 PCa patients, 37 BPH patients and 24 healthy individuals. Written informed consents were obtained from all patients and healthy individuals. The level of miR-145 in urinary EVs were significantly increased in patients with PCa compared with the patients with BPH. Significant increases were observed in miR-145 levels when patients with Gleason score ≥ 8 tumours compared with Gleason score ≤ 7. The same tendency were found in paired serum EVs samples. Receiver-operating characteristic curve revealed that miR-145 in urinary EVs combined with PSA could differentiate PCa from BPH better than PSA alone (AUC 0.863 and AUC 0.805 respectively). In serum EVs, all of these 5 miRNAs were significantly higher in patients with PCa than with BPH.
Conclusion: HFD was appropriate for urinary EVs miRNA analysis when compared with conventional UC. Urinary EVs miR-145 is upregulated from PCa patients compared BPH patients and healthy controls. We suggest the potential use of urinary EV miR-145 as a biomarker of PCa.

OF15.02

Novel platform for extracellular vesicle mRNA characterisation and mutation detection in cancer patient blood

Zhaogang Yang[1], Xinmei Wang[1], Kwang J. Kwak[2], Jiaming Hu[1] and L. James Lee[3]

[1]The Ohio State University, OH, USA; [2]Chemical and Biomolecular Engineering at Ohio State University, OH, USA; [3]Chemical and Biomolecular Engineering

Introduction: Extracellular vesicles (EVs) contain proteins and RNAs that can affect the recipient cells and serve as biomarkers for diseases. Non-coding microRNAs in EVs have been studied extensively, however, the characterisation of EV-mRNAs remains challenging due to their extremely low expression and the fragmentation of mRNAs in EVs. Therefore, novel techniques that can detect the mRNA fragments in EVs at high sensitivity and specificity are needed. Here,we aim to develop a novel biochip for the detection of EV-mRNAs and their mutations in cancer patient blood.
Methods: We designed new toehold-initiated molecular beacons (Ti-MBs) that are much more stable and sensitive than conventional hairpin molecular beacons (Co-MBs) and can detect mRNA targets with a single-base mis-match. Those Ti-MBs are encapsulated in cationic lipoplex nanoparticles (CLNs) and tethered on the surface of a thin glass, which can capture individual EVs in plasma via EV-CLN fusion and identify EV mRNA targets via MB-mRNA hybridisation using a high resolution fluorescence microscope in a single step. Well defined plasma samples from lung cancer, liver cancer and pancreatic cancer patients were tested.
Results: Comparing to qRT-PCR, our tethered lipoplex nanoparticle (TLN) biochip is much more sensitive for EV mRNA detection, requires smaller sample size (20 μL), uses less assay time (<4 h), and can detect single-point mutated mRNAs in EVs. We examined a glucose regulation gene, transketolase 1 (TKTL1), and thyroid transcription factor 1 (TTF1), a well-known upregulated mRNA in lung cancer tissue, to demonstrate the applicability of TLN biochip in non-small cell lung cancer (NSCLC) detection. We also examined two genes related to hepatocellular carcinoma (HCC), alpha fetoprotein (AFP) and glypican-3 (GPC3), for liver cancer detection. Those 2-mRNA classifiers can distinguish cancer patients from healthy individuals with high accuracy, not achievable by any existing methods. Our TLN biochips with Ti-MBs can also identify EGFR mutations in lung cancer and KRAS mutations in pancreatic cancer via EVs in cell line culture medium or patient plasma without qRT-PCR amplification and gene sequencing.
Conclusion: Our TLN biochip may serve as a platform for EV capture and characterisation of mRNAs and mutations in cancer patient blood.

OF15.04

Extracellular RNA is promising biomarker for eary detection of cancers

Yukie Nishiyama[1], Yumiko Koui[2], Yuki Yamamoto[1], Genki Nishimura[1], Masaki Kinehara[1], Akira Shimamoto[1], Morito Okada[2] and Hidetoshi Tahara[1]

[1]Department of Cellular and Molecular Biology, Hiroshima University Institute of Biomedical & Health Sciences; [2]Department of Surgical Oncology, Hiroshima University, Hiroshima, Japan

Introduction: Extracellular vesicles (EVs) including exosomes released into the extracellular environment from a variety of cells, and can be used for cell-to-cell communication *in vivo*. It is well known that circulating RNA and exRNA are powerful tool for cancer biomarker. We focused on exRNA and circulating RNA using serum for cancer biomarker.
Methods: Cell were cultured with DMEM with FBS and the cell supernatant were collected without FBS medium. Extracellular vesicles (EVs) were purified by ultracentrifugation or sucrose gradient fractionation. The size and amount of isolated EVs were measured by qNano (iZON). Circulating RNA is purified using miRNeasy Mini Kit (Qiagen). Next generation sequencing (NGS) is performed using IonPGM and IonS5 (Thermo Fisher Scientific Inc.). All of the patients provided written informed consent to participate in the study (Approved by IRB committee in Hiroshima university).
Results: We identified several microRNAs biomarker specific for pancreatic cancer, head and neck cancer and breast cancer using serum and plasma. We also identified cancer specific pre-miRNAs, pri-miRNA and isomiR which is distinguish between cancer and healthy volunteer. It is known that isomiRs are not caused by RNA degradation during sample

preparation for NGS. Some of isomiR profiling is well correlated in exRNA profiling in cultured EVs from cancer cell lines. Therefore, isomiR alterations in circulating RNA should be powerful and significant tools to identify the origin and the type of cancers.
Conclusion: We believe that our NGS platform based biomarker discovery may provide the useful information to use for early detection, prognosis and companion diagnosis in cancers.

OF15.05

Extracellular vesicle mRNA and miRNA characterisation in ovarian cancer ascites and peritoneal fluid

Cindy Yamamoto[1], Taku Murakami[1], Melanie Oakes[1], Michael Muto[2], Ross Berkowitz[2] and Shu-Wing Ng[2]

[1]Hitachi Chemical Co. America, Ltd. R&D Center; [2]Brigham and Women's Hospital, MA, USA

Ovarian cancer has the highest mortality rate of all gynaecological cancers worldwide, partly due to the lack of early signs or symptoms leading to diagnosis at relatively advanced stages for this disease. Our goal was to determine if potentially novel biomarkers could be identified for early screening using ovarian cancer ascites extracellular vesicles (EVs). Here, we describe characterisation of ovarian cancer ascites and peritoneal fluid EVs and detection of specific mRNA and miRNA. Fluids were collected from subjects with benign cysts, endometrioma, or low/high grade serous ovarian carcinoma. EVs isolated from these fluids were found to be EpCAM positive by ELISA and have concentrations greater than 2.0×10^{10} particles/mL by nanoparticle tracking analysis. Particle sizes from peritoneal fluids were 158.7 ± 28.3 nm while ascites were 87.3 ± 18.0 nm ($p < 0.05$). Using a 96-well exosome collection filterplate, both peritoneal fluids ($n = 10$) and ascites fluids ($n = 8$) were processed in parallel and subsequently, qPCR screening of 34 mRNA and 18 miRNA was performed. These studies identified five and six significantly differentially expressed normalised EV mRNA and miRNA ($p < 0.05$), respectively. At least one of these markers was shown to be present in healthy plasma (n = 3) and significantly increased in conditioned media of SKOV3 and OVCAR3, which are high-grade serous ovarian cancer cell lines compared respectively to immortalised ovarian surface and fallopian tube epithelial cells, the hypothesised cells of origin for ovarian cancer development. Further studies are necessary to determine if this marker is differentially expressed in ovarian cancer plasma. EVs may provide a potentially novel source for discovery of biomarkers for early detection of ovarian cancer.

OF15.06

Characterisation of exosomes and exosomal circular RNA from pancreatic ductal adenocarcinoma carcinoma cell lines

Keith Laderoute[1], Daniel Renouf[2], David Shaeffer[2], Marcel Bally[3], Emma Guns[4] and Jessica Kalra[3]

[1]SRI, Inc.; [2]Pancreas Centre BC; [3]BC Cancer Research Center, British Columbia, Canada; [4]Vancouver Prostate Center, Vancouver, Canada

Introduction: Pancreatic ductal adenocarcinoma (PDAC) continues to demonstrate poor outcomes due to its late stage of diagnosis. Research has concentrated on finding biomarkers for early detection while the cancer is still localised and amenable to therapy, however, these markers remain elusive. Exosomes are quickly becoming a prominent tool in biomarker research, and PDAC exosomes are showing promise in the development of liquid biopsies for early screening programmes. The studies described focus on characterising exosomes collected from the conditioned media of PDAC cell lines as well as inventorying the RNA contents of these extracellular vesicles. We are particularly interested in exploring a novel class of non-coding RNA, circular RNA (circRNA) for our studies. We believe that aberrantly expressed genes in PDAC produce different types of circRNAs that become enriched in tumour-secreted exosomes.
Methods: Exosomes were isolated from a normal pancreatic exocrine cell line (htert-HPNE) as well as three PDAC cell lines ranging from well to poorly differentiated, including PANC-1, BxPC3and MIAPaCa-2. The size and relative abundance of exosomes was quantified by transmission electron microscopy (TEM) and nanotracker analysis (NTA). Circular RNA was purified from exosomes (exo-circRNA) and used to construct RNA-Seq libraries. Characteristics of exosomes and exo-circRNA comparisons were made between cell lines.
Results: Exosome size ranged from 40 nm to 160 nm. The smallest structures were observed from the PANC-1 cell line and concentrations varied with the lowest abundance coming from HPNE and MiPaCa cells. CircRNAs in exosomes were easily isolated from all 4 cell lines, and comparative RNA-seq analyses revealed a number of interesting circRNA species that show cell line specificity.
Conclusions: The studies described demonstrate that specific circRNAs can be readily extracted from the exosomes secreted into the conditioned media of PDAC cell lines. We hope that this novel tool can be further developed to help to diagnose pancreatic carcinoma when it is amenable to surgical resection and/or chemotherapy, thereby reducing the mortality associated with this disease.

OF15.03

In vivo characterisation of EV miRNA secretion into cerebrospinal fluid (CSF) by glioblastoma

Johnny C. Akers, Valya Ramakrishnan, Bob S. Carter and Clark C. Chen

Center for Theoretical and Applied Neuro-Oncology, University of California, San Diego, CA, USA

Introduction: Glioblastoma is the most common form of primary brain neoplasm and remains one of the deadliest of human cancers. Robust platform for minimally invasive biomarkers that would allow assessment of tumour burden or therapeutic response remains an unmet clinical need. While efforts to analyse clinical cerebrospinal fluid (CSF) for such biomarkers are ongoing, initial efforts were plagued by heterogeneity in patient demographics, characteristics, and variation in sample acquisition. Here we establish a murine model for *in vivo* characterisation of CSF changes that occur secondary to glioblastoma growth.
Methods: Patient derived glioblastoma line expressing was orthotopically implanted into nude mice. 4 weeks after injection, brain tissue and murine CSF from the cisterna magna were collected from tumour-bearing mice and age-matched, mock injected nude mice. We modified a PCR method designed to assess RNA derived from single cells to characterise miR-21 level in CSF.
Results: In glioblastoma xenograft specimens, miR-21 was expressed at levels 10–160 fold higher than that seen in murine brain. There was a >10 fold increase in the CSF miR-21 level of mice with glioblastoma tumour relative to those that underwent mock injection. The level of CSF miR-21 did not directly correlate with glioblastoma tumour size, suggesting potential influences of microenvironment factors in this process. While miR-16 and miR-10b were similarly elevated in glioblastoma xenograft specimens, we did not detect increased levels of these miRNAs in xenograft bearing mice relative to the mock injected mice.
Conclusion: Our results suggest that glioblastoma selectively export miRNAs through EV secretion *in vivo*. The model established here lays the foundation for interpretation of clinical CSF data as well as future mechanistic studies of EV transport between anatomic compartments.

Room: Metropolitan Ballroom West and Centre
Symposium Session 16 – EV Omics
Chairs: Juan Falcon-Perez and Suresh Mathivanan 3:45–5:15 p.m.

LBO.09

Extracellular vesicles containing Chs3 and Fks1 rescue cell wall defective yeast and protect from antifungal agents

Kening Zhao, Mark Bleackley, Marilyn Anderson and Suresh Mathivanan

La Trobe Institute for Molecular Science, Melbourne, Australia

Introduction: Though most of the knowledge pertaining to Endosomal Sorting Complex Required for Transport (ESCRT) machinery interactions were obtained from yeast, very little is known about their role in extracellular vesicle (EVs) biogenesis in yeast. Furthermore, it is unclear whether EVs have any role in cell wall remodeling.
Methods: EVs were isolated using differential centrifugations from various ESCRT knockout yeast strains. Protein quantification, electron microscopy, nanoparticle tracking analysis, quantitative proteomics and carbohydrate analysis were done to characterize these EVs. Yeast cells were treated with specific drugs to enrich for different EV types. Survival assays were carried out with EVs and antifungals.
Results: A series of 10 yeast knockout strains including Vps2âˆ†, Vps23âˆ†, Vps36âˆ†, Bro1âˆ†, Hse1âˆ†, Fks1âˆ†, Chs3âˆ†, Atg8âˆ†, Mrpl32âˆ† and Mst27âˆ† were established. Characterization and quantitative proteomic analysis revealed that ESCRT knockout and cell wall mutant EVs were altered in terms of protein amounts, morphologies, size and protein cargo compared to WT. Carbohydrate analysis of EVs revealed enrichment of glucose and mannose in Bro1âˆ† and Hse1âˆ† EVs. In spite of 85% proteome coverage in EVs, ESCRT components were found to be significantly depleted in yeast EVs. These results suggest that yeast EVs are significantly different from mammalian exosomes. Proteomic analysis highlighted the enrichment of cell wall remodeling enzymes, glucan synthase Fks1 and chitin synthase Chs3, especially in Vps2âˆ† and Vps23âˆ† EVs. To understand whether yeast EVs can remodel the cell wall, functional uptake assays were performed with WT and cell wall mutant (Chs3âˆ†) strains. Interestingly, EVs were able to protect WT and cell wall mutant strains from antifungal caspofungin and the plant defensin NaD1. However, EVs from Fks1âˆ† or Chs3âˆ† were unable to rescue the yeast cells from antifungals. Furthermore, the protection from antifungals were abrogated when EVs from Chs3âˆ† Vps23âˆ† double knockout strain were incubated with yeast cells. Co-culture of Chs3âˆ† strain expressing GFP and WT or Vps23âˆ† strain increased Chs3âˆ† strain survival upon caspofungin treatment.
Summary/Conclusion: Overall, we were able to confirm that yeast EVs are different from mammalian exosomes. Secondly, EVs with cell wall remodeling enzymes were able to rescue yeast from antifungal agents.

OF16.01

Differences and similarities in full-length and fragmented non-coding RNA biotypes in EV from differentially stimulated dendritic cells

Tom A.P. Driedonks[1], Susanne G. van der Grein[1], Yavuz Ariyurek[2], Henk P. J. Buermans[2], Henrike Jekel[1], Franklin W.N. Chow[3], Amy H. Buck[3], Marca H.M. Wauben[1], Peter-Bram A.C. 't Hoen[4] and Esther N.M. Nolte-'t-Hoen[1]

[1]Department of Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [2]Leiden Genome Technology Centre, Leiden University Medical Centre, Leiden, The Netherlands; [3]Institute of Immunology and Infection Research, Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh, UK; [4]Department of Human Genetics, Leiden University Medical Centre, Leiden, The Netherlands

Introduction: The presence and function of miRNA in extracellular vesicles (EVs) have been widely studied. However, the majority of EV RNA consists of other small RNA types such as tRNA, Y-RNA, SRP-RNA, Vault RNA, snoRNA and snRNA. Fragmented forms of these RNAs have been proposed to exert gene regulatory functions. It is unknown if and how incorporation of these RNAs in EVs is regulated, and how they function in EV-mediated communication. We differentiated dendritic cells (DC) into immunogenic and tolerogenic phenotypes, which release EVs that differently affect T cell responses. We investigated which RNA types were consistently present in EVs and which types were differentially incorporated depending on the signal imposed on DC.
Methods: EVs released by *in vitro* cultured DC that were left unstimulated or differentiated into highly immunogenic or tolerogenic phenotypes were isolated using differential centrifugation and density gradient purification. Deep sequencing was performed on small RNA (15–300 nt) isolated from EVs and parent cells (n = 3). Observations were validated by Northern blot or RT-qPCR.
Results: miRNA were underrepresented in EVs compared to cells, but the miRNA content showed large differences between immunogenic and tolerogenic EVs. snoRNA and snRNA were underrepresented in EVs but were highly similar between the two conditions. tRNA were highly abundant and enriched in EVs compared to cells, but no major differences were found between immunogenic and tolerogenic EVs. Interestingly, tolerogenic and immunogenic EV differed in levels of Y-RNA and incorporated Y-RNA fragments. Importantly however, Northern blot showed a very different full length:fragments ratio for tRNA and Y-RNA than expected based on sequencing data.
Conclusion: Differentiation signals imposed on dendritic cells affect the miRNA and Y-RNA content of released EVs, while other non-coding RNA types remain largely unchanged. This suggests that RNA types other than miRNA potentially contribute to EV function.

OF16.02

CD63, MHC class 1 and CD47 identify subsets of extracellular vesicles containing distinct populations of micro-RNA

Sukhbir Kaur[1], Abdel G Elkahloun[2], Anush Arakelyan[3], Tim G Myers[4], Otaizo-Carrasquero Francisco[4], Weiwei Wu[5], Leonid Margolis[3] and David D Roberts[1]

[1]Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; [2]Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA; [3]Eunice-Kennedy National Institute of Child Health and Human Development; [4]Genomic Technologies Section, Research Technologies Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; [5]Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

Recent publications have identified complex functions of extracellular vesicles (EVs) in mediating cell-cell communication. Some of these functions are mediated by intercellular transfer of mRNA, miRNA and other small RNAs that post-transcriptionally alter the transcriptome of target cells. RNA sequencing of EVs derived from cancers or biological fluids from patients to identify disease-specific bioactive RNAs is also of increasing diagnostic interest. However, the heterogeneity in sizing, density, and composition of EVs has limited progress towards understanding their functions and diagnostic utility. CD63 and MHC-1 have been used as markers to purify EVs, but it is unclear whether EVs expressing different markers differ functionally. We and others have identified CD47 on EVs and shown that its presence on EVs can alter their functional signalling in target cells. To further investigate the functional heterogeneity of EVs, we have captured EVs from Jurkat T cells and colon carcinoma cells using CD47, CD63 and MHC-1 antibodies and evaluated each subset using flow cytometry and miRNA expression analysis. EVs expressing CD47, CD63 and MHC class I differ

in their size distribution and miRNA content based on RNA sequencing. EVs captured by each marker also differed from EVs lacking the respective markers. Marker-specific sorting of some miRNAs into EVs was conserved between T cells and colon carcinoma cells. Our results suggest that CD63+, MHC-1+, and CD47+ EVs of EVs, contain distinct but overlapping populations of miRNAs. Our findings also suggest that EVs exhibit functional heterogeneity, and specific surface biomarkers may be useful to identify EVs with specific functions and to enrich disease-specific EVs from liquid biopsy.

OF16.03

miRNAs enclosed in small extracellular vesicles are selectively secreted and retained in cellular senescence and modulate keratinocyte functionality

Lucia Terlecki Zaniewicz[1], Vera Pils[1], Julie Latreille[2], Ingo Lämmermann[1], Madhusudhan Reddy Bobbili[3], Regina Weinmüllner[1], Dietmar Pum[4], Matthias Hackl[5], Michael Mildner[6], Frederique Morizot[2], Florian Gruber[1,6] and Johannes Grillari[1]

[1]Christian Doppler Laboratory for Biotechnology of Skin Ageing, Department of Biotechnology, BOKU University Vienna, Austria, [2]Department of Skin Knowledge and Women Beauty, Chanel R&T, Pantin, France; [3]University of Natural Resources and Life Sciences, Department of Biotechnology; [4]University of Natural Resources and Life Sciences, Institute of Nanobiotechnology; [5]TAmiRNA GmbH; [6]Department of Dermatology, Medical University of Vienna, Austria

The senescence-associated secretory phenotype (SASP) is one hallmark of senescent cells, and characterised by the secretion of pro-inflammatory factors that alter the tissue microenvironment. Recently, miRNAs packaged into extracellular vesicles (EV-miRNAs) have been found as part of intercellular communication. Here, we investigated whether miRNAs, especially those enclosed in small EVs might also be part of the SASP and if specific miRNAs are preferentially secreted or retained after entry into cellular senescence. Therefore, small EVs of stress-induced premature senescent (SIPS) and quiescent control cells (Q) were harvested by differential centrifugation. We observed a fourfold higher increase of exosome-like vesicles in SIPS cells and consequently and elevated abundance of almost all miRNAs. Correlation of intra- and extracellular miRNA abundance indicated a selective packaging mechanism and identified prominent candidates that might be able to confer a biological role on recipient cells. Furthermore, to test if EV-miRNAs are part of the paracrine crosstalk between fibroblasts (HDF) and keratinocytes (NHEK), we confirmed uptake of c.elegans specific cel-miR-39 enclosed in EVs derived from HDF by NHEK in monolayers and in *in-vivo* mimicking skin-equivalents. Finally, we evaluated how sEVs derived from senescent HDF influence differentiation potential and wound healing capacity of NHEK and identified miR-23a as a crucial mediator of the miR-SASP. To summarise, our data indicate that EV-miRNAs of senescent fibroblasts are *bona fide* members of the SASP and suggest the term "miR-SASP". The selective sorting of specific senescence-associated EV-miRNAs contributes to the communication between fibroblasts and keratinocytes in 2D and 3D human skin models. However, the underlying specific molecular mechanism and the biological role of other highly abundantly and selectively secreted SA-miRNAs, such as miR-23a, and their implications in ageing and age-associated diseases remains to be determined.

OF16.04

Molecular lipidomics of urinary exosomes: can molecular lipid species serve as cancer biomarkers?

Skotland Tore[1], Ekroos Kim[2], Kauhanen Dimple[3], Simolin Helena[3], Seierstad Therese[4], Berge Viktor[5], Sandvig Kirsten[1] and Alicia Llorente[6]

[1]Department of Molecular Cell Biology, Institute for Cancer Research, Oslo University Hospital-The Norwegian Radium Hospital, Oslo, Norway; [2]Lipidomics Consulting; [3]Zora Biosciences; [4]Division of Radiology and Nuclear Medicine, Oslo University Hospital, Oslo, Norway; [5]Oslo University Hospital, Oslo, Norway; [6]Oslo University Hospital-The Norwegian Radium Hospital, Oslo, Norway

Introduction: The protein and nucleic acid composition of urinary exosomes has been extensively characterised during the last decade and several exosomal proteins and nucleic acids have been identified as biomarkers for several diseases. There is however limited information about the lipid composition of urinary exosomes. We have here performed a mass spectrometry study to reveal the lipid composition of urinary exosomes and investigated the potential use of lipid species as prostate cancer biomarkers.
Methods: Urinary exosomes were isolated by sequential centrifugation and characterised by electron microscopy, nanoparticle tracking analysis and western blot to analyse their quality/purity. Then, a high-throughput mass spectrometry quantitative lipidomic analysis was performed to characterise their molecular lipid composition.
Results: The lipid composition of exosomes isolated from urine samples of healthy individuals was first analysed. Over 100 lipid species were quantified in urinary exosomes. The comparison of urinary exosomes and cell line derived-exosomes revealed several differences between the two exosome populations, for example, in cholesterol and phosphatidylcholine. Then, the lipid composition of 15 prostate cancer patients and 13 healthy controls were analysed. Several lipids species were found to be significantly different when the two groups were compared. The highest significance was shown for phosphatidylserine (PS) 18:1/18:1 and lactosylceramide (d18:1/16:0). Furthermore, combinations of these lipid species and PS 18:0–18:2 were shown to have high sensitivity and specificity for prostate cancer.
Conclusion: This study shows that lipids in urinary exosomes are promising prostate cancer biomarkers. Moreover, it also shows the importance of *in vivo* studies for biomarker studies.

OF16.05

Mining the new human reference interactome to investigate interaction-mediated protein sorting into extracellular vesicles

Dae-Kyum Kim[1], Katja Luck[2], Dong-Sic Choi[3], Hanane Ennajdaoui[1], Atina G. Cote[1], Ghazal Haddad[4], Jochen Weile[1], Fan Yang[1], Dayag Sheykhkarimli[1], Kerstin Spirohn[2], Luke Lambourne[5], Human Reference Interactome Team[1], Jan Tavernier[6], David E. Hill[2], Tong Hao[2], Marc Vidal[2], Janusz Rak[7], Michael A. Calderwood[2] and Frederick P. Roth[1]

[1]Donnelly Centre, University of Toronto, Toronto, Canada; [2]Center for Cancer Systems Biology (CCSB) and Department of Cancer Biology, Dana-Farber Cancer Institute; [3]The Research Institute of the McGill University Health Centre, Montreal, Canada; [4]Department of Molecular Genetics, University of Toronto, Toronto, Canada; [5]Department of Bioengineering, McGill University, Montreal, Canada; [6]Department of Medical Protein Research, VIB; [7]The Research Institute of the McGill University Health Centre, Montreal, Canada

Communication among cells and their environment is pivotal for their survival and function. Extracellular vesicles (EVs) have drawn great interest as one mean of cell-cell communication. However, the repertoire of proteins sorted into EVs is incompletely known, as is the mechanism of protein sorting. Recruitment to EVs is frequently accomplished by protein-protein interaction (PPI). Unfortunately, current knowledge of PPIs is greatly biased by gene popularity and expression level. Here, we assembled information on the EV-related proteome, and analysed it in the context of a new human reference interactome (HuRI) with ~50,000 interactions derived from Y2H screening of ~300 M pairs amongst ~17.5 K human protein-coding genes. Pairs of EV-associated proteins found in the same experimental conditions are more likely to interact physically according to HuRI, which supports a role for PPIs in EV protein-sorting. HuRI interactions within the EV proteome showed 6 clusters, each centred on a well-connected "hub" protein. Of these six, three (TSG101, HGS and ACTB) were previously known to be important in EV biogenesis, more specifically, TSG101 and HGS for exosome and ACTB for ectosome biogenesis. The other three – KRT, HNRNPK and SGTA – represent new candidate EV-sorting proteins. The mRNA-binding protein HNRNPK is a candidate for acting both in protein and mRNA sorting into EVs. KRT, which was considered as an artefact of contamination, seems to be important for EV biogenesis. The six corresponding lists of protein partners may represent distinct classes of EV-sorted proteins or EVs themselves. Thus, systematic evaluation of EV-associated PPI interactions yields new clues for classification and protein sorting mechanisms of extracellular vesicles.

OF16.06

In-depth proteomics of cancer-associated fibroblasts secretome and role of exosomes in tongue cancer progression

Simona Principe[1], Salvador Mejia-Guerrero[1], Vladimir Ignatchenko[1], Ankit Sinha[2], Keira Pereira[2], Laurie Ailles[1] and Thomas Kislinger[1]

[1]Princess Margaret Cancer Centre, Toronto, Canada; [2]Department of Medical Biophysics

Introduction: Bidirectional communication between cells and their microenvironment is crucial for both normal tissue homeostasis and for tumour growth. During the development of tongue cancer (TC), cancer-associated fibroblasts (CAFs) create a supporting niche by maintaining a bidirectional crosstalk with cancer cells, mediated by classically secreted factors and nanometre-sized vesicles, called exosomes. Little is known about the activation of normal fibroblasts into CAFs and what determines CAFs unique functional properties. Therefore, a better understanding of CAF-derived exosomal cargo and its functional effects on tongue cancer cells are required. This will provide novel insights on the complex molecular interactions underlying stromal-tumour crosstalk and help to elucidate their roles in regulating carcinogenesis.

Methods: To better elucidate the role of CAFs in the tumour stroma and how secreted proteins contribute to TC progression, we have isolated nine matched pairs of human primary fibroblasts from resected tumours (CAFs) and adjacent tissue (AFs) and characterised them according to established CAF markers. We employed shotgun proteomics to comprehensively characterise CAFs secretome in order to: (1) evaluate the effect of CAFs conditioned media and exosomes on TC cells; (2) identify CAF-associated proteins and investigate their roles as potential biomarkers using richly annotated tissue microarrays (TMA).

Results: We have generated a comprehensive dataset of 4247 proteins which represents a detailed signature of a pro-tumorigenic stroma. First we show the different characteristics and effects of CAFs-secreted fractions (exosomes and conditioned media) on TC cells growth and migration. Next, we perform quantitative proteomics to highlight CAF-enriched proteins and identify candidates specific to the *CAF-like* state. We identify one novel secreted CAF protein involved in TC progression and currently investigate its use as a prognostic biomarker using a 90 patient TMA.

Summary: We use an in-depth proteomic approach to characterise the complexity of CAF secreted factors and evaluate the effects of CAF exosomes on tumour progression. Our data provides a comprehensive resource that can be used to identify CAF-enriched proteins and novel exosomal cargo with functional relevance in TC.

Room: Metropolitan Ballroom East
Symposium Session 17 – EVs in Tissue Repair and Inflammation
Chairs: Chris Gardiner and Shilpa Buch 3:45–5:15 p.m.

LBO.10

The role of platelet-derived extracellular vesicles in the GPIb-dependent adhesion of monocytes in models of thrombo-inflammation

Aigli Evryviadou[1], Myriam Chimen[1], Clare Box[2], Matthew Harrison[3], Sahithi Kuravi[1], Holly Payne[1], Dean Kavanagh[1], Steven Thomas[1], Neena Kalia[1], Alexander Brill[4], Steve Watson[1], Paul Harrison[5], Gerard Nash[1] and Ed Rainger[1]

[1]Institute of Cardiovascular Sciences, University of Birmingham, United Kingdom; [2]Institute of Cancer and Genomic Sciences, University of Birmingham, United Kingdom; [3]Mars Petcare; [4]Institute of Cardiovascular Sciences; [5]Institute of Inflammation and Ageing, University of Birmingham, United Kingdom

Introduction: Our previous studies had identified a novel pathway where monocytes could bind to platelets adherent to appropriately activated endothelium in a model of vascular inflammation. Given this observation, we wondered whether formation of platelet-monocyte aggregates in blood might also support the thrombo-inflammatory recruitment of monocytes to the vessel wall.
Methods: We employed FACS, confocal microscopy, *in vitro* flow assays and intravital microscopy in order to carry out our studies.
Results: Upon addition of platelet stimulants to blood, we assessed binding of platelets to leukocytes by measuring acquisition of the platelet-specific marker GPIb. Heterotypic aggregate formation was time-dependent and largely monocyte-specific. Monocytes accumulated GPIb in quanta significantly lower than that on a single platelet, suggesting that monocytes acquired GPIb from platelet-derived extracellular vesicles. Provision of pre-stained platelet-derived extracellular vesicles in blood also resulted in rapid accumulation of GPIb specifically on monocytes with similar dynamics. Confocal microscopy demonstrated abundant GPIb-positive particles, sized in the sub-micron range in the cytoplasm of monocytes.
The GPIb delivered to monocytes was functional in *in vitro* flow assays, as it enhanced monocyte adhesion to immobilized recombinant vWF, or to TGF-β-stimulated endothelial cells. In order to test this function *in vivo* we used a transgenic strain of mice in which human IL4-R is expressed under the GP1bα promoter. This allows endogenous platelets to be cleared using an anti-human IL4R antibody. Using intravital microscopy of the cremaster circulation which had been stimulated by the topical application of TGF-β1, we observed adoptively transferred monocytes decorated with platelet microvesicles were recruited and rolled on the vessel wall in a GPIb-dependent manner.
Summary/Conclusion: Thus, we describe a novel role of platelet-derived extracellular vesicle accumulation by monocytes. This thrombo-inflammatory pathway of monocyte recruitment may be important in vascular disease, as it is likely to bypass the usual regulatory pathways that control monocyte recruitment during inflammation.
Funding: This work was funded by the British Heart Foundation.
Symposium Session 18 – 3:30 pm

OF17.01

Osteoblast-derived extracellular vesicles represent a novel and highly potent method for stimulating bone formation

Owen Gareth Davies[1,2], Sophie C. Cox[2], Mark P. Lewis[1] and Liam M. Grover[2]

[1]Loughborough University, Loughborough, United Kingdom; [2]University of Birmingham, United Kingdom

Introduction: Over 300,000 osteoporosis-related fragility fractures are reported annually in the UK with an associated cost of over £1.5 billion. These numbers are expected to double by 2020, putting tremendous strain on the healthcare system. Over the last decade, considerable attention has been focussed on cell-based approaches to solve this problem. Although these methods have yielded promising results, their translation is frequently hindered by insurmountable regulatory and ethical hurdles. By harnessing the regenerative capacity of extracellular vesicles (EVs) we have developed an acellular yet biological therapy able to regenerate bone.
Methods: EVs were isolated from mineralising murine osteoblasts using ultracentrifugation and profiled using atomic force microscopy (AFM), direct light scattering (DLS), transmission electron microscopy (TEM) and ImageStream flow cytometry. Their effects on MSC osteogenic differentiation were assessed against the clinical gold-standard, BMP-2. MSC osteogenesis was analysed using alizarin red calcium staining, alkaline phosphatase (ALP) quantification, and PCR. Mineral phase and quality was determined using X-ray fluorescence (XRF) and infrared spectroscopy (IR). LC-MS/MS was used to define the EV proteome and raw data files processed using MaxQuant. MS/MS spectra were searched against the mouse proteome and analysed using Gene Ontology Enrichment analysis.
Results: EVs ($CD9^+/CD63^+/CD81^+$) of ~160 nm were able to significantly enhance ALP levels, mineralisation rate and mineral volume beyond the current gold-standard, BMP-2. XRF elemental mapping identified considerable co-localisation of calcium and phosphorus. IR analysis of the mineral phase confirmed the presence of brushite, a mineral only stable at more acidic pH conditions, such as those found in multivesicular bodies (MVBs). The presence of amide peaks indicative of collagen were also distinguished when compared with the control. Proteomic analysis of EVs revealed the presence of collagens and extracellular binding proteins associated with osteogenesis.
Conclusion: Our data suggests that EVs function to enhance MSCs capacity to utilise local calcium/phosphates. As such, they hold considerable potential as an acellular yet biological approach to regenerative medicine.

OF17.02

Myofibroblast-derived extracellular vesicles promote epithelial cell senescence in idiopathic pulmonary fibrosis

Tsukasa Kadota[1], Yusuke Yoshioka[1], Yu Fujita[2], Jun Araya[2], Kazuyoshi Kuwano[2] and Takahiro Ochiya[1]

[1]Division of Molecular and Cellular Medicine, National Cancer Centre Research Institute, Japan; [2]Division of Respiratory Disease, Department of Internal Medicine, The Jikei University School of Medicine, Japan

Introduction: Idiopathic pulmonary fibrosis (IPF) is a chronically progressive and lethal fibrosing interstitial lung disease of unknown aetiology. Aberrant phenotypic alterations of alveolar epithelial cell, including accelerated cellular senescence, have been proposed to be responsible for regulating fibrosis development. However, the mechanisms leading to the cell senescence are poorly understood. Here, we investigated the involvement of extracellular vesicles (EVs)-mediated intercellular communication between lung fibroblasts (LFs) and primary human bronchial epithelial cells (HBECs) in regulating epithelial cell senescence during IPF pathogenesis.
Methods: LFs were obtained from IPF and non-IPF patients who underwent lobectomy. EVs from LFs were isolated by ultracentrifugation and characterised by electron microscopy, western blot and nanoparticle tracking analysis. The profiles of EV microRNAs (miRNAs) were examined by microarray. Cellular senescence was evaluated with senescence-associated β-galactosidase staining and expression levels of $p21^{Waf1/Cip1}$ and $p16^{INK4A}$. LFs stimulated with TGF-β1 were used as an *in vitro* model of myofibroblast differentiation.
Results: LF-derived EVs were characterised by the presence of EV marker proteins such as CD9 and CD63. EVs derived from IPF-LFs and TGF-β-treated LFs demonstrated decrease in particle counts and

protein levels as compared with those derived from non-IPF and non-TGF-β-treated LFs. Confocal microscopic examination elucidated uptake of labelled EVs derived from LFs by HBECs. Intriguingly, EVs derived from TGF-β-treated LFs and IPF-LFs promoted cellular senescence in HBECs. EV microarray analysis elucidated that several miRNAs were downregulated in EVs derived from IPF-LFs, which negatively regulate cellular senescence.
Conclusion: IPF-LFs may accelerate epithelial cell senescence via EV secretion as a part of aberrant mesenchymal-epithelial interactions in IPF pathogenesis.

OF17.03

Intranasal A1-exosomes decrease inflammation and preserve neurogenesis in the hippocampus as well as prevent memory dysfunction after status epilepticus

Qianfa Long[1,2], Dinesh Upadhya[1,2], Bharathi Hattiangady[1,2], Dong-Ki Kim[1], An Su Yeon[1], Bing Shuai[1,2], Darwin J. Prockop[1] and Ashok K. Shetty[1,2]

[1]Institute for Regenerative Medicine, Texas A&M University College of Medicine, College Station, TX, USA [2]Olin E. Teague Veterans' Medical Center, Temple, TX, USA

Introduction: Status epilepticus (SE), a medical crisis that is typically terminated through antiepileptic drug treatment, leads to hippocampus dysfunction typified by neurodegeneration, inflammation, altered neurogenesis as well as cognitive and memory deficits. Here, we examined the effects of intranasal (IN) administration of extracellular vesicles (EVs) secreted from the human bone marrow derived mesenchymal stem cells on SE-induced adverse changes. The EVs employed in this study are $CD9^{-}CD63^{+}CD81^{+}$ and referred to as A1-exosomes because of their robust anti-inflammatory properties (1).
Methods: We subjected young mice to pilocarpine induced SE for 2 h and then intranasally administered A1-exosomes or vehicle twice over 24 h.
Results: Intranasally administered A1-exosomes invaded the cerebral cortex and reached the hippocampus within 6 h of administration and animals receiving them exhibited diminished loss of glutamatergic and gamma-aminobutyric acid-ergic neurons, and greatly reduced inflammation in the hippocampus. Moreover, the neuroprotective and anti-inflammatory effects of A1-exosomes were coupled with the long-term preservation of normal hippocampal neurogenesis and cognitive and memory function, in contrast to waned and abnormal neurogenesis, persistent inflammation and functional deficits in animals receiving vehicle.
Conclusion: These results provide the first evidence that IN administration of A1-exosomes is efficient for minimising the adverse effects of SE in the hippocampus and preventing SE-induced cognitive and memory impairments.

Acknowledgments: Supported by Emerging Technology Funds from the State of Texas, a Merit Award from the VA (I01 BX002351) and an NIH grant (P40OD11050).

Reference

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OF17.04

PDGF enhances the pro-regenerative properties of EVs released form adipose stem cells

Tatiana Lopatina[1], Andrea Ranghino[1], Massimo Cedrino[1], Chiara Gai[1], Renato Romagnoli[2], Maria Felice Brizzi[1] and Giovanni Camussi[1]

[1]Department of Medical Sciences, University of Turin, Torino, Italy; [2]Liver Transplant Unit, Azienda Ospedaliera Città della Salute e della Scienza, University of Turin, Torino, Italy

Introduction: Adipose mesenchymal stem cells (ASCs) promote angiogenesis and tissue regeneration through paracrine mechanisms. We have previously shown that platelet derived growth factor (PDGF) stimulate ASC to secret EVs (PDGF-EVs) with a stronger pro-angiogenic potential than EVs secreted in basic conditions (bEVs). The aim of the present study was to investigate the molecular mechanism involved in angiogenic and immunomodulatory activity of PDGF-EVs.
Methods: For this purpose we studied *in vitro* the effects of PDGF-EVs on the secretion of inflammatory factors by peripheral blood mononuclear cells (PBMCs) as well as their influence on PBMC adhesion on endothelial cells (EC). bEVs were used for comparison. *In vivo* we have also studied the effects of bEVs and PDGF-EVs in an acute limb ischemia pre-clinical model. The molecular differences between bEVs and PDGF-EVs were also investigated.
Results: bEVs but not PDGF-EVs stimulated secretion of IFNg, IL-1 and TNFa by PBMCs while secretion of IL-10 was significantly enhanced after stimulation with PDGF-EVs. The adhesion of PBMCs to EC was enhanced by bEVs, but not by PDGF-EVs. In addition, PDGF-EVs were able to stimulate nitric oxide production in EC. *In vivo* results demonstrate that PDGF-EVs was significantly more effective in restoring large vessel reperfusion and in inhibiting muscle damage and inflammatory cell recruitment than bEVs. PDGF-EV proteomic analysis demonstrated differences in pro-angiogenic and pro-inflammatory protein content when PDGF-EVS and bEVs were compared. In particular PDGF-EVs were enriched in HGF, TGFa/b and their receptors, IL-1 ra, VEGF, Tie, OSM, uPA, uPAR, MMPs, thrombospondins, BDNF, ICAM, IGF. While bEVs carried high levels of IFN-γ, G-CSF, GM-CSF and CD40/TNFRSF5. PDGF-EVs were also enriched in pro-regenerative microRNAs, such as miR-130a, miR-19a, miR-296, miR-17, miR-21, miR-92a, miR-34b, miR-520d, miR-100, miR-146b and long non-coding RNA such as MALAT1.
Conclusion: This study demonstrates that PDGF stimulates ASCs to secrete EVs enriched in anti-inflammatory and pro-regenerative factors, which could account for PDGF-EV-mediated protection against ischemia reperfusion injury.

OF17.05

VEGF-induced damage of glomerular endothelial cells in Alport syndrome: effect of amniotic fluid stem cell-derived extracellular vesicles

Laura Perin[1], Sargis Sedrakyan[1], Stefano Porta[2] and Benedetta Bussolati[3]

[1]Children's Hospital Los Angeles, CA, USA; [2]University of Torino, Italy; [3]Department of Molecular Biotechnology and Health Sciences, University of Torino, Italy

Introduction: Injection of amniotic fluid stem cells (AFSC) delays the course of progression of renal fibrosis in animals with Alport syndrome, enhancing kidney function and improving survival. The mechanisms responsible for these protective outcomes are still largely unknown. We here evaluated whether extracellular vesicles produced by the AFSC (AFSC-EVs) could be responsible for the observed renoprotection.
Methods: Glomerular endothelial damage was assessed by histology and electron microscopy. AFSC (1×10^6) and deriving EVs (30 mg) were administered by single intracardiac injection. Levels of proteinuria and of intraglomerular VEGF were also analysed. AFSC-EVs were isolated by ultracentrifugation, characterised for the expression of angiogenic surface markers by FACS and for the presence of angio-modulating microRNAs by RT-PCR. VEGFR1 knockout AFSC were generated by transfection and deriving EVs isolated. VEGFR1 neg and $VEGFR1^{+}$ EVs were tested in an *in vitro* model of endothelial cytotoxicity induced by high VEGF doses.
Results: Alport mice were characterised by glomerular endotheliosis, proteinuria and increased glomerular VEGF levels. Intra-ventricularly injected AFSC showed strong modulation of glomerular endothelial cell damage in Alport mice and reduced the elevated VEGF signalling and proteinuria. Similar effects were obtained by a single injection of AFSC-derived EVs. AFSC-EVs expressed VEGFR1 and VEGFR2, and contained angio-modulatory microRNAs. *In vitro*, AFSC-EVs prevented the cytotoxic effect of high VEGF levels. This was due to VEGF trapping through its binding to the EV surface VEGFR1, as shown by immunoprecipation analysis. In contract, VEGFR1 knockout EVs failed to show endothelial protection, thus indicating that VEGF trapping is a potentially viable mechanism for AFSC mediated renoprotection in mice.
Conclusion: Taken together our findings identify a new mechanism of action, i.e. VEGF trapping, by AFSC-EVs suggesting that AFSC-EVs could target a specific signalling pathway within the glomerulus thus representing a new potential glomerulus-specific targeted intervention.

Room: Harbour Ballroom
Symposium Session 18 – Biogenesis: EVs and Viruses
Chairs: Leonid Margolis and Jennifer Jones 3:45–5:15 p.m.

LBO.11

Host exosomes released during infection with Rift Valley fever virus play a protective role by destroying the virus cell reservoirs and by inhibiting viral replication and release

Ramin M. Hakami[1], Noor Ahsan[2], Gavin Sampey[3], Benjamin Lepene[4], Robert Barclay[3], Sergey Iordanskiy[3] and Fatah Kashanchi[3]

[1]School of Systems Biology and NCBID, George Mason University; [2]George Mason University; [3]Laboratory of Molecular Virology, George Mason University, Manassas, Virginia, USA; [4]Ceres Nanosciences Inc., Manassas, Virginia, USA

Introduction: Our laboratory studies exosome (EX) effects during infection with highly pathogenic biodefense agents such as Rift Valley fever virus (RVFV). RVFV has been classified as a pathogen of highest concern (Category A) that causes a devastating zoonotic disease and has the potential to be used for bioterrorism. There are no approved vaccines or therapeutics available. Intriguingly, unlike exosome studies reported for several other viral infections, the EXi released during RVFV infection play a protective role for the host.
Methods: EX were purified from both naïve Vero cells (EXu) and infected Vero cells (EXi) by serial centrifugation followed by sucrose density gradient purification, and characterized by TEM and Western analysis. Plaque assays were performed on purified exosome fractions to demonstrate that they are free of virus particles. In addition, clones infected with RVFV that remained viable (resistant clones) were generated and shown not to release virus, and exosomes released from these cells were also isolated and characterized. Both naïve immune and non-immune recipient cell types were treated with EXi or EXu (as control) and analyzed for effects on viability. Effects of pre-treatment with EXi on virus replication and release were also analyzed. qRT-PCR was performed on biological replicates of EXi to determine whether they contain viral genome. Furthermore, using both Western and mass spectrometry analyses of four biological replicates, the viral protein content of EXi were analyzed.
Results: Our results demonstrate that although immune cell types (T-cells and monocytic cells) stay viable after infection with RVFV they show a drastic rate of apoptosis through PARP cleavage and caspase 3 activation following treatment with EXi, a novel mechanistic finding for RVFV infection. Furthermore, pre-treatment with EXi followed by RVFV infection significantly reduces virion production and release. The EXi carry all three viral RNA genome segments (L, M, and S) and also the viral envelope glycoprotein and the viral nucleocapsid protein.
Summary/Conclusion: As it has been proposed that RVFV uses immune cells as replication reservoirs, our results present a model in which the released EXi act to combat infection in two ways, by targeting the RVFV cellular reservoirs for destruction and also by interfering with viral replication and release.

OF18.01

Virosomes: the interplay between viral infection and exosome production

Robert Barclay[1], Catherine DeMarino[1], Angela Schwab[1], Michelle Pleet[1], Gavin Sampey[1], Sergey Iordanskiy[1], Ramin M. Hakami[2], Benjamin Lepene[3], Nazira El-Hage[4] and Fatah Kashanchi[1]

[1]Laboratory of Molecular Virology, George Mason University, Manassas, VA, USA; [2]School of Systems Biology and NCBID, George Mason University, VA, USA; [3]Ceres Nanosciences Inc., Manassas, VA, USA; [4]Department of Immunology, Herbert Wertheim College of Medicine, Miami, FL, USA

Introduction: HIV infection results in a chronic illness since long-term HAART can lower viral titers to an undetectable level. However, discontinuation of therapy rapidly increases virus burden. Moreover, patients under HAART frequently develop various metabolic disorders, neurocognitive abnormalities and cardiovascular diseases.
Methods: We use a combination of ultracentrifugation and nanoparticle capture to concentrate our EVs from various bodily fluids for downstream assays.
Results: We have previously shown that exosomes containing transactivating response (TAR) element RNA enhance susceptibility of undifferentiated naïve cells to HIV infection. Up to a million copies of TAR RNA/mL were also detected in the serum from HIV infected humanised mice suggesting that TAR RNA may be stable *in vivo*. We recently have found another viral non-coding RNA that we termed TAR-*gag* which does not code for a protein, but is present in the exosomes. Incubation of exosomes from HIV-1 infected cells with primary cells resulted in a dramatic increase of pro-inflammatory cytokines, IL-6 and TNF-β, indicating that exosomes containing TAR RNA could play a direct role in control of cytokine gene expression. Furthermore, the single stranded 5′ or 3′ processed stem RNA binding to TLRs activates the NF-κB pathway and regulates cytokine expression. In our most recent data, we find that the exosomes from infected cells are increased in numbers when cells are treated with specific antiviral drugs or innate immune molecules such as IFN-a. These findings suggest that while the virus is being suppressed (specifically or non-specifically), the amount of exosomes that contain viral products increase after treatment.
Conclusion: Our results directly indicate that HIV viral release and exosome release have overlapping biogenesis pathways including the ESCRT pathway. Similar results are also seen from other neuro-tropic RNA viral infections including HTLV, Ebola, RVFV, and Zika infection which will be discussed. Our data implies that exosomes from virally infected cells under either specific or non-specific treatment (i.e. latent cells) control immune cells survival and pathogenesis. Therefore, targeting these particles may be a method to lower overall viral burden in infected immunocompromised hosts.

OF18.02

Attempts to re-define cellular components specifically incorporated in HIV as compared to sEVs and exosomes secreted by infected cells

Lorena Martin-Jaular[1], Zhaohao Liao[2], Pehuen Pereyra Gerber[3], Matias Ostrowski[3], Kenneth Witwer[2] and Clotilde Théry[4]

[1]Institut Curie, Paris, France; [2]The Johns Hopkins University School of Medicine, MD, USA; [3]INBIRS Insitute, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina; [4]Institut Curie, PSL Research University, INSERM U932, Paris, France

Introduction: HIV, exosomes and/or other small extracellular vesicles (sEVs) share biogenesis aspects and physicochemical characteristics, making their separation difficult. Some cellular proteins are described as excluded from virions (e.g. CD45), whereas others are incorporated (e.g. CD63). We re-evaluated these results in light of our recent demonstration that many subtypes of sEVs are co-isolated by a protocol of EV isolation similar to that used for HIV isolation, and of our recently published sets of protein combinations distinguishing exosomal and non-exosomal sEVs (1). Our goal is to obtain HIV-free sEVs to allow assessing their functional properties.
Methods: Medium of Jurkat cells infected or not with VSV-G–pseudotyped NL4-3-IRES-EGFP was subjected to differential centrifugation, and velocity top-to-bottom iodixanol gradient was used to separate sEVs from virus in the 100,000*g* pellet (100 K). Gradient fractions

were analysed by WB for the presence of different markers and by AChE assay.
Results: Differential centrifugation showed that CD45 is more abundant in large/medium EVs than in sEVs from both uninfected and infected cells. Velocity gradients revealed at least two types of sEVs in the 100 K pellet. Fractions from the top of the tube contained CD9 and some CD45 but little or no CD63 (i.e. non-exosomal sEVs), whereas intermediate fractions contained CD9, CD63, and syntenin-1, hence probably exosomes. Gag and CD63 but little or no CD9, Syntenin-1 and CD45 were detected in bottom fractions of infected cells' 100 K pellet. Importantly, AChE activity was found in fractions different from those enriched in Gag but also from those enriched for the other sEVs/exosome markers.
Conclusions: Despite exclusion from virus containing fractions, neither AChE activity nor CD45 are satisfying markers to distinguish HIV from exosomes. Velocity gradients achieve some separation of sEVs/exosome or virus markers, but overlap of distribution makes it difficult to use them for unbiased proteomic comparisons. Further work will be required to identify, if they exist, sEV and/or exosomal components specifically excluded from HIV virions.

Reference

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OF18.03

Picornavirus infection induces the release of distinct EV populations containing infectious virus and altered host-derived contents

Susanne G. van der Grein[1], Kyra A.Y. Defourny[1], Huib H. Rabouw[2], Martijn A. Langereis[2], Frank J.M. van Kuppeveld[2] and Esther N.M. Nolte-'t-Hoen[1]

[1]Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [2]Department of Infectious Diseases and Immunology – Virology division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Introduction: Picornaviruses are classically believed to release non-enveloped progeny through the induction of cell lysis, yet were recently shown to also exit from intact cells inside extracellular vesicles (EVs). Enclosure of virus particles inside EVs may have a large impact on viral dissemination or antiviral immunity and therefore on the pathology of many infectious diseases. To better understand the function of picornavirus-induced EVs we performed in-depth analysis of host- and virus-derived components enclosed in these EVs and their release dynamics during infection.
Materials and Methods: EVs released by pre-lytic picornavirus-infected cells were separated into subpopulations using differential ultracentrifugation and density gradient purification. EV and viral particles were quantified using high-resolution flow cytometry and end-point titration, and viral or host-derived EV contents were analysed by western blot and qPCR.
Results:We found that early after viral infection, before cell lysis occurs, picornavirus triggers the release of several distinct EV populations. Small EVs pelleted at 100,000*g* and floating to low-density fractions contained mature infectious viral particles. In addition, EV pelleted at 10,000*g*, which likely represent larger EV, also enclosed viral particles. Early after infection these virus-containing EVs constitute a prominent portion of the released infectious particles, and their contribution to infectivity decreases over time. Interestingly, prior to the release of virus-containing EVs, picornavirus also induces secretion of EV lacking viral products but with altered host components.
Conclusion: Picornavirus infection induces major changes in the repertoire of EVs released by cells. Moreover, the release dynamics of virus-containing EVs and other virus-induced EVs is tightly regulated. These different EV types may each play a distinctive role in virus propagation or host protection, contributing to the continuous battle between virus and host.

OF18.04

Withdrawn at author's request.

OF18.05

Extracellular vesicle cargo delivery through membrane fusion: regulation by factors that promote and restrict enveloped virus cell entry

Michael Hantak, Enya Qing and Thomas M. Gallagher

Loyola University Chicago, IL, USA

Introduction: Extracellular vesicles (EVs) facilitate intercellular communications by transferring membrane-bound and cytosolic factors between cells. Delivery of these factors into target cells requires fusion of EV and cell membranes. Enveloped viruses also deliver their internal cargo through membrane fusion. We hypothesised that EVs and enveloped viruses are similarly regulated at the level of membrane fusion.
Methods: EV-directed cargo delivery was measured using a membrane fusion-dependent reporter complementation assay. EVs were loaded with luciferase fragments, and then applied to target cells containing complementary luciferase fragments. Fusion between EV and target cell membranes permitted fragment complementation, which generated quantifiable luciferase levels. Using this assay, we determined whether known regulators of enveloped virus membrane fusion also controlled EV-cell fusion. We also determined whether EV subtypes vary in their capacity to mediate EV-cell fusion and subsequent cargo delivery.
Results: EVs definitively brought reporter cargoes into target cells through a membrane fusion process. EV-mediated membrane fusion was restricted by the anti-viral interferon-induced transmembrane protein 3 (IFITM3), and was promoted by the pro-viral transmembrane protease serine 2 (TMPRSS2). Both IFITM3 and TMPRSS2 incorporated into EV particles. Their incorporation required the ESCRT machinery of EV-producing cells. Functional ESCRT machinery was also required for EV-directed cargo transfers.
Conclusions: Cytoplasmic cargoes are primarily transferred by ESCRT-generated EVs. These ESCRT-generated EVs are regulated by at least two factors, IFITM3 and TMPRSS2, which restrict and promote cargo delivery, respectively. These two factors are found on the EVs. These findings are consistent with the hypothesis that EVs and enveloped viruses have strikingly similar cargo delivery mechanisms.

OF18.06

Extracellular vesicles and lipoproteins influence cellular response to HIV-1 Infection

Lisa Learman[1], Zhaohao Liao[1], Bonita Powell[1], Dillon Muth[1], Carol Cooke[1], Erez Eitan[2] and Kenneth Witwer[1]

[1]The Johns Hopkins University School of Medicine, MD, USA; [2]National Institute on Aging, National Institutes of Health

Introduction: Cells grown in serum-containing, EV-depleted (EVD) media display decreased proliferation and viability. We recently reported both increased release and infectivity of HIV-1 from cells grown in EVD media. Here, we interrogate effects of EV depletion on HIV-1 susceptibility. We also examine the possibility that standard EV depletion protocols affect non-EV particles.
Methods: Media were prepared with EVD FBS (Thermo Fisher), with fewer particles per unit volume than FBS prepared in-house by various methods as assessed by nanoparticle tracking analysis and protein assays for EV markers. The HeLa-derived HIV-1 reporter line TZM-bl, encoding luciferase under the control of the HIV-1 promoter, was grown in media containing 10% EVD or non-depleted FBS. Cells were exposed to titrated HIV-1, and susceptibility to infection was quantified by luciferase assay. Add-back experiments were done with purified EVs and density gradient fractions of lipoprotein particles. Transmission electron microscopy and lipid assays assessed purified serum components. Statistics were done for matched samples and corrected as appropriate for multiple tests (significance: alpha < 0.05).
Results: Cells grown in EVD media were significantly more susceptible to infection by each of two strains of HIV-1, with interaction of passage number and sensitivity. Like others, we noticed LDL and HDL depletion by EVD protocols: EV depletion was accompanied by depletion of lipoproteins, and EM of gradient-purified LDL and HDL revealed EV-

like particles. Therefore, in addition to EV add-back, we examined the effects of LDL and HDL on HIV-1 susceptibility of cells in depleted media. Added to depleted cells 2 h before infection, physiologically relevant levels of neither LDL nor HDL affected HIV-1 susceptibility. However, with 24 h pre-treatment, LDL but not HDL partially restored baseline levels of susceptibility in a dose-dependent manner.

Conclusions: Cellular lipid sensing may influence susceptibility to HIV-1 infection, but EVs are not the only contributors. LDL depletion by standard EVD protocols also contributes to increased HIV-1 susceptibility. Even density gradient-based purification methods may not completely separate EVs from lipoproteins, complicating assessment of physiological roles of these particles.

Poster Session F01 – EV-Based Cancer Biomarkers
Chairs: Malene Jorgensen and Kwang-Pyo Kim 5:15–6:30 p.m.

PF01.01

Gamma-glutamyltransferase activity in exosomes as a marker for prostate and renal cell cancers

Masafumi Ito[1], Kyojiro Kawakami[1], Kengo Horie[2], Yasunori Fujita[1], Yoko Matsuda[3], Tomio Arai[3], Koji Kameyama[2], Taku Kato[2], Koichi Masunaga[4], Yutaka Kasuya[4], Masashi Tanaka[5], Takashi Deguchi[2] and Kosuke Mizutani[2]

[1]Research Team for Mechanism of Ageing, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan; [2]Department of Urology, Gifu University Graduate School of Medicine, Gifu, Japan; [3]Department of Pathology, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan; [4]Department of Urology, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan; [5]Department of Clinical Laboratory, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan

Introduction: Exosomes have the potential as a marker for various diseases including cancer. In the present study, we performed proteomic analysis of exosomes isolated from prostate cancer (PC) cell lines and identified a candidate marker. We then examined the usefulness of the exosomal marker in patients with renal cell cancer (RCC) as well as PC.
Methods: Exosomes isolated from LNCaP PC cell line and its sublines C4, C4-2 and C4-2B were subjected to proteomic analysis. GGT (gamma-glutamyltransferase) activity was measured using a fluorescent probe, g-glutamyl hydroxymethyl rhodamine green. Immunohistochemical analysis of tissue specimens was performed with anti-GGT1 antibody.
Results: Among proteins upregulated in C4-2 and C4-2B cells than in LNCaP cells, we focused on GGT1, a cell-surface enzyme that regulates the catabolism of extracellular glutathione. The levels of GGT1 large and small subunits were elevated in exosomes isolated from C4-2 and C4-2B cells by differential centrifugation and immunocapture. In cell lysates and exosomes, GGT1 expression correlated with GGT activity. In human serum, size exclusion chromatography demonstrated the presence of GGT activity and GGT1 subunits in fractions positive for CD9 and density gradient centrifugation revealed the co-presence of GGT1 subunits with CD9. Since GGT activity correlated with GGT1 expression in serum exosomes isolated by differential centrifugation, we measured serum exosomal GGT activity in patients. We found that serum exosomal GGT activity was significantly higher in PC patients than in benign prostatic hyperplasia (BPH) patients. In support of this finding, immunohistochemical analysis showed increased GGT1 expression in PC tissues compared with BPH tissues. In RCC, we also found that serum exosomal GGT activity was significantly increased in late-stage patients than in early-stage patients.
Conclusion: Our results suggest that serum exosomal GGT activity could be a useful marker for PC and RCC.

PF01.02

Isolation and characterisation of small RNAs in the extracellular vesicles from washed stool samples for colorectal cancer diagnosis

Tae-Young Roh, Seung-ick Oh, Seokjin Ham, Hyungjoo Ko, Insoon Jang and Sookil Tae

Pohang University of Science and Technology, Pohang, Republic of Korea

Introduction: The presence of nucleic acid in the extracellular vesicles (EVs) has been known for its role in the intercellular communication. The EVs are exfoliated from cells and can be detected in diverse sources such as tissues, blood, urine and stools. Here, we examined the existence of small RNAs including miRNAs in EVs of the washed stool from colorectal cancer patients and analysed them as potential biomarkers.
Methods: The EV was isolated from washed stool of colorectal cancer patients by using the aqueous two-phase system (ATPS) and its RNA was purified by treating with TRIzol. The total small RNAs including miRNAs were purified and analysed by small RNA-seq using next-generation sequencing (NGS) technology.
Results: From comparative analysis of normal and colorectal cancer patient samples, we could identify tens of novel small RNAs as well as known miRNAs. The potential applicability of novel small RNA was further explored.
Conclusion: For the non-invasive diagnosis of colorectal cancer, the RNAs surrounded and protected the the EVs are useful targets for the development of biomarkers. We examined the small RNAs in the EVs of the washed stool from colorectal cancer patients. Some novel small RNAs could be used as biomarker for colorectal cancer biomarkers with further massive examination of clinical samples.

PF01.03

Phenotype analysis of extracellular vesicles secreted by pancreatic cancer cell lines: exposition of EpCAM, Glypican-1 or Phosphatidylserine

Etienne Buscail[1], Sandrine Dabernat[1], Olivier Degrandi[1], Celine Gounou[2], Sisareuth Tan[2] and Alain R. Brisson[2]

[1]INSERM 1035, Bordeaux, France; [2]UMR-5248 CNRS, University of Bordeaux, Bordeaux, France

Introduction: No reliable biomarker is yet available to diagnose pancreatic cancer (PaCa). Extracellular vesicles (EVs) secreted by tumour cells are attracting interest as potential biomarkers (1). However, caution is warranted in considering EV literature, due to the difficulty of EV characterisation and isolation. This study aims at describing the size, phenotype and concentration of EVs released by four PaCa cell lines, using immuno-cryo-electron microscopy (EM), flow cytometry (FC) and biochemistry (2,3). We focused on EVs exposing EpCAM, antigen overexpressed in various carcinomas, glypican-1 (GPC-1), a proposed marker of PaCa (1), and phosphatidylserine (PS), a procoagulant lipid with potential role in cancer-related thrombosis.
Methods: EVs were isolated from CaPan2, BXPC3, PanC1 and MiaPaCa-2 cells by low-speed centrifugation or with the total exosome isolation kit (Life Technologies). EpCAM+, GLPC-1+ and PS+ EVs were detected by FC using antibodies (Abs) against EpCAM (Novel Life Tech), GLPC-1 (Thermo Fisher, ATLAS) or annexin-5 (Anx5)[3]. For immuno-cryo-EM, gold particles were conjugated with anti-EpCAM or anti-GLPC-1 Abs or Anx5[2].
Results: By FC, we found that large amounts of EpCAM+ EVs were released by CaPan2 and BXPC3 cells, and significantly less (>100×) by PanC1 and MiaPaCa-2 cells. In addition, larger amounts (~4×) of PS+ EVs were released by PanC1 and MiaPaCa-2, as compared to CaPan2 and BXPC3 cells. No GPC-1+ EVs were detected with the two Abs used here in the four cell lines.
By immuno-cryo-EM, we identified EpCAM+ EVs in CaPan2 and BXPC3 supernatants. These EVs ranged in size from 100 nm to 1 µm. Most EpCAM+ EVs of small size (<100 nm), the so-called exosomes, were also labelled by Anx5. No labelling was observed with the anti-GPC-1 Abs used. Western-blotting experiments revealed the presence of GPC-1 in cell extracts for the two Abs. This suggests that GPC-1 proteins are present in the cell cytoplasm, but not at the EV surface.
Conclusion: This study provides a semi-quantitative analysis of EVs secreted by PaCa cell lines, and is currently complemented by a study of EVs present in plasma of PaCa patients.

References

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PF01.04

Surface glycosylation profiling of evs using lectin-nanoparticles

Parvez Syed[1], Laura Lehtinen[2], Kamlesh Gidwani[1], Khirul Islam[3], Janne Leivo[4], Kim Pettersson[3] and Urpo Lamminmäki[3]

[1]Department of Biochemistry/Biotechnology, University of Turku, Finland; [2]Department of Pathology, University of Turku and Turku University Hospital, Turku, Finland; [3]Department of Biochemistry, Division of Biotechnology, University of Turku, Finland; [4]Department of Urology, Erasmus Medical Centre, Rotterdam, The Netherlands

Introduction: Extracellular vesicles (EVs) are secreted by almost all cells and present variety of proteins, lipids and glycans on their surface. The majority of the surface tumour markers reported to date are either glycoproteins or glycolipids. Traditionally, the EV-surface glycosylation profiling is either done using mass spectrometry or lectin microarrays. However, both these methods require isolation of EVs. In this study, we use lectins, which bind to the glycan part of the glycoproteins, conjugated with Eu^{3+}-doped nanoparticles (NP) to identify the glycans presented on the surface of the EVs.
Methods: The EVs from cell-free cell culture supernatants of HEK293 and ovarian cancer (OvCa) cell lines (SKOV3, M022 and M019i) were captured using biotinylated anti-CD63 antibody immobilised onto streptavidin coated 96-well plate. The captured EVs were probed with lectins conjugated with ~100 nm-sized polystyrene NPs containing 30,000 Eu^{3+} ions. The lectins used in this study were, galactose binding Macrophage Galactose Lectin (MGL) fused with mouse FC, mannose-binding dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and mannose-binding lectin (MBL) and β-galactoside-binding galectin-4. Anti-CD63 antibody conjugated NPs were also used to validate the efficacy of the assay to capture the EVs. Detection of the signals (λ_{ex}: 340 nm, λ_{em}: 615 nm) was done using Victor 2, 1420 multilabel counter (Perkin-Elmer).
Results: The assay involving biotinylated anti-CD63 antibody and anti-CD63-NPs showed the presence of EVs in all the cell culture supernatants. Similarly, the signals were also obtained mannose binding lectin-NPs (DC-SIGN-NP and MBL-NP). This confirms the presence of mannose containing glycoproteins, which has been reported in several studies, on the surface of the EVs. However, the signals from MGL-mouse FC-NP and galectin-4-NP were obtained only OvCa EVs but not from HEK293 EVs.
Conclusion: This preliminary study shows the presence of galactose and β-galactoside containing glycans only on the surface of EVs derived from OvCa cell lines but not on the surface of the EVs from HEK293. Such approach can further be exploited for diagnosis of various cancers by identifying the differently glycosylated patterns on the surface of the EVs obtained from the biofluids.

PF01.05

miRNA profiling in uveal melanoma exosomes as a metastatic risk biomarker

Kyra N. Smit[1], Natasha van Poppelen[2], Taral Lunavat[3], Kasper Derks[4], Rob Verdijk[5], Hanneke Mensink[6], Jan Lötvall[7], Annelies de Klein[1] and Emine Kilic[2]

[1]Department of Clinical Genetics, Erasmus MC Rotterdam; [2]Department of Ophthalmology, Erasmus MC Rotterdam; [3]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden; [4]Department of Clinical Genetics, Maastricht UMC, Maastricht; [5]Department of Pathology, ErasmusMC Rotterdam; [6]Oogziekenhuis Rotterdam; 7Krefting Research Centre, University of Gothenburg, Sweden

Introduction: Uveal melanoma (UM) is the most common primary intraocular malignancy in adults. It is a highly aggressive cancer in which nearly 50% of patients die from liver metastasis. UM patients can be divided into three groups based on their genetic profile and disease free survival, the low risk, intermediate risk and high risk tumours. Recently, we have shown that high risk patients can be identified based on the expression of five miRNAs. Since tumour tissue is not always available and biopsies are not without risk, it is important to develop a method that can identify high risk patients in a non-invasive manner. Exosomal miRNAs are an excellent candidate for this application. Our aim is to analyse the presence of our marker miRNAs in UM exosomes.
Methods: Exosomes were isolated from the cell culture medium of a non-metastatic and high risk metastatic UM cell line by ultracentrifugation and were characterised by western blot, electron microscopy and Nanosight tracking analysis (NTA). RNA was isolated from exosomes by the Qiagen RNeasy micro kit and quantified by an Agilent bioanalyzer. Subsequently, miRNA expression was measured by Taqman miRNA qPCR assays.
Results: All exosome samples isolated from cell medium showed expression of CD81. Bioanalyzer confirmed the absence of ribosomal RNA and an abundance of small RNAs. qPCR analysis shows changes in the expression of some classifier miRNAs in exosomes extracted from cell lines, demonstrating that the classifier miRNA signature in UM cells partially overlaps with the miRNA signature in exosomes secreted by UM cells.
Conclusion: These exosomal miRNAs show great promise as a reliable predictor of disease free survival in UM. Next goal is to detect these exosomal markers in blood of UM patients since this will enable us to provide all UM patients with a prognosis.

PF01.06

Analysis of extracellular vesicle-derived RNAs isolated with the Vn96 peptide from human renal and bladder cancer cell lines

Naoufal El Bekkouri[1,2], Simi Chacko[1], Darwin D'Souza[1], Sebastien Fournier[1], Annie-pier Beauregard[1], Sami Benzina[1], Awanit Kumar[1], Sandra Turcotte[3], Anirban Ghosh[3], Nicolas Crapoulet[4], Stephen M. Lewis[3] and Rodney J. Ouellette[3]

[1]Atlantic Cancer Research Institute, New Brunswick, Canada; [2]Université de Moncton, New Brunswick, Canada; [3]Department of Chemistry and Biochemistry, Université de Moncton, New Brunswick, Canada; [4]Department of Chemistry and Biochemistry, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Canada

Introduction: Renal cell carcinoma (RCC) and bladder cancer (BC) have rising incidence and high rates of recurrence. Unfortunately, conventional diagnostic methods are far from adequate, as cytology lacks sensitivity and biopsy is an invasive procedure. There is an unmet need for accurate, minimally invasive biomarkers to support clinical decision-making. Extracellular vesicles (EVs) are nanosized membrane-bound vesicles that mediate cell-cell communication. Due to the stability of EV-derived RNAs (EV-RNAs) in body fluids and the functional implication of non-coding RNA molecules in the tumour microenvironment, EV-RNAs have been a subject of great interest in recent years, especially in the context of "liquid biopsy" and circulating biomarkers. The aim of this study is to investigate novel minimally invasive biomarkers for RCC and BC.
Methods: EVs released from nine cell lines were isolated using the Vn96 affinity capture peptide, then characterised by nanoparticle tracking analysis (NTA), western blot (WB) and Agilent technologies. We have used transcriptome sequencing (RNA-seq) to investigate the EV-RNAs.
Results: NTA, WB and RNA profiles confirmed the presence and the purity of EVs in all cell lines. High-throughput RNA-seq revealed differences in the RNA species content between cellular and EV-RNAs. We have derived an EV-RNA expression signature for RCC and BC. These signatures are based on statistically significant differences in expression levels and profiles in tumour-derived EV-RNAs versus normal cell lines EV-RNAs. Interestingly, we found altered expression of miRNAs and lncRNAs that are known to act in epithelial-to-mesenchymal transition and angiogenesis in tumour-derived EV-RNAs. Moreover, certain genes (GAPDH and miR16) are consistently present at similar levels in all EV-RNA samples and conditions tested, suggesting that these genes may be reliable internal standards.
Conclusion: Our RNA-seq data offers a catalogue of EV-RNAs for renal and bladder cancer cell lines. This initial screening "*in vitro*" forms the basis for validation of EV-RNA expression signatures in biological fluids of patients with RCC or BC. Further mechanistic studies are needed to understand the functional involvements of EV-RNAs in RCC and BC pathogenesis.

PF01.07

Exosomes as biomarkers in paediatric acute lymphocytic leukaemia

Shabirul Haque and Sarah Vaiselbuh

The Feinstein Institute for Medical Research at Northwell Health, NY, USA

Introduction: Exosomes are secreted by most cells including tumour cells in biological fluids. Because exosomes are easily accessible by liquid biopsy and carry the genetic fingerprint of parental cells, exosomes emerge as promising biomarkers in cancer diagnostics. Although many hurdles such as high throughput methods for exosome isolation and poor exosomal

RNA recovery remain to be solved, clinical applications of exosomes as biomarkers will certainly gain momentum in this rapidly expanding field.
Objective: To identify leukaemia-specific exosomes harvested from conditioned medium (CM) of ALL cell lines as well as from serum of P-ALL patients that correlate with disease status.
Methods: Exosomes were isolated from healthy donor (HD), P-ALL patient serum and from conditioned medium (CM) of JM1, SUP-B15 and CL-01 human cell lines by ultracentrifugation. Exosomal identity was confirmed with NanoSight Tracking Analysis as well as by Western Blot. We used an innovative method for direct conversion of very small starting volumes of purified exosomes into cDNA followed by gene amplification by two-step PCR. Gene amplification was confirmed on 1.5% agarose electrophoresis.
Results: CM-exosomes of JM1 and SUP-B15 tested positive for CD10/CD34 expression by 2-Step PCR in contrast to CL-01 cells (control) that were negative. In addition, we evaluated serum exosomes in duos of liquid biopsies for either CD10/CD34 or CD10/CD19 expression (according to phenotypic expression of the parental leukaemic blasts). P-ALL exosomes at Day 1 (diagnosis) tested positive for CD10/CD34 or CD10/CD19 while P-ALL exosomes of the same patients at Day 29 (remission) became negative. Similarly, serum-derived exosomes from P-ALL relapse patients (blast count in bone marrow aspirates range 60–85% by FCM) were positive in contrast to P-ALL-exosomes of the same patients at time of 1st remission (blast count 0%) that tested negative. HD exosomes (controls) tested negative for CD34 expression.
Conclusion: P-ALL exosomes secreted in serum can be detected by gene expression analysis for leukaemia-specific markers CD10/CD34 or CD10/CD19 and may serve as leukaemia biomarkers that correlate with disease status in the bone marrow. These preliminary data need validation in larger cohort clinical biomarkers studies.

PF01.08

Novel tissue- and cancer-specific markers identified by proteomic profiling of extracellular vesicle cargo

Stephanie N. Hurwitz, Mark A. Rider, Joseph L. Bundy, Xia Liu, Rakesh K Singh and David G. Meckes

Florida State University College of Medicine, FL, USA

Introduction: Circulating extracellular vesicles (EVs) hold great potential for use in minimally-invasive disease detection, including cancer diagnostics. Accumulating evidence has shed light on differences in EV biogenesis and content across cells of various origins.
Methods: Here, we analyse and compare the secretion and content of EVs from cancer cells and non-tumorigenic cells using nanoparticle tracking and mass spectrometry. We further characterise conserved EV proteins by density gradient purification of vesicle sub-populations.
Results: We previously conducted a global proteomic profile of EV content across 60 cancer cell lines derived from nine histological types compiled by the National Cancer Institute (NCI-60), identifying 6071 proteins with 213 common to all isolates. Cargo found to be differentially expressed among EVs from varying origins offer potential for cancer diagnosis and prognostic monitoring. Here we provide new evidence of novel breast cancer biomarkers by comparison of cancer cell-derived EV content to protein cargo in EVs released by non-tumorigenic cells. Additionally, examination of common EV cargo revealed sub-population specific markers of EVs, providing improvement to current EV classification strategies.
Conclusion: Tumorigenic and non-tumorigenic cells may be distinguished based on their diverse EV profiles, and differences in content of EVs may present novel diagnostic tools for cancer detection. On the other hand, common EV proteins across cells likely reflect key players in EV subpopulation biogenesis. The findings in this study contribute to understanding the underlying mechanisms of EV formation and offer promising targets for cancer diagnosis.

PF01.09

Detection of exosomal microRNA using molecular beacon for cancer diagnosis

Jeong Ah Kim[1], Ji Hye Lee[2] and Won Jong Rhee[2]

1Biomedical Omics Group; 2Incheon National University

Exosomes are small extracellular vesicles that contain biomarker miRNAs produced from their originating cells and travel through the circulatory system. Exosomal miRNA from the body fluids has been investigated as an attractive biomarker in the diagnosis of disease. However, current techniques, including real-time PCR analysis are still unsuitable for the *in situ* detection of exosomal miRNA due to their time-consuming and laborious process. In this study, we have developed a novel diagnosis system that enables *in situ* single step detection of miRNA in a whole exosome for applying to various diseases. We have demonstrated that miRNAs in exosomes can be detected directly using a nano-sized oligonucleotide probe, molecular beacon (MB). MiR-21 in exosomes from breast cancer cells were detected successfully by molecular beacons in a quantitative manner. We tested exosomes that originated from different types of cells including MCF-7 breast cancer cell line to determine whether MBs bind to miR-21 with high specificity. We also investigated whether MB is delivered into exosomes by going through the exosomal membrane and discussed whether permeabilisation treatment can be used to improve the delivery of MBs inside exosome, giving a high level of hybridisation. This method is simple, fast, and sensitive, so it will offer great opportunities for the high-throughput diagnosis and prognosis of diseases.

PF01.10

Multiplexed detection of exosome microRNAs using molecular beacons

Jin Hee Lee[1], Jeong Ah Kim[2] and Won Jong Rhee[1]

[1]Incheon National University, Incheon, Republic of Korea; [2]Biomedical Omics Group

Multiplexed detection of miRNAs in an exosome is developed, which can be utilised as a PCR-free efficient diagnosis method for various diseases. Exosomes are small extracellular vesicles that contain biomarker miRNAs from their originating cells. Because they circulate throughout bodily fluids, exosomal biomarkers offer great advantages for diagnosis in many aspects. In general, PCR-based methods can be used for exosomal miRNA detection but they are laborious and time-consuming, which make them unsuitable for high-throughput diagnosis of diseases. Herein, we show that multiple miRNAs can be detected simultaneously in exosomes using miRNA-targeting oligonucleotide probes, molecular beacons. Exosomes from MCF-7 were used for the production of exosomes because MCF-7 has a high level of miR-21, miR-27a and miR-375. Each molecular beacons successfully hybridised with multiple miRNAs in the cancer cell-derived exosomes even in the presence of high human serum concentration. The proposed method described in this article is beneficial to high-throughput analysis for disease diagnosis, prognosis, and response to treatment because it is a time-, labour-, and cost-saving technique.

PF01.11

Del-1 promotes proliferation and migration of tamoxifen-resistant MCF7 cells

Soo jung Lee[1], Ho Yong Park[2], Jae-hwan Jeong[1], Byung Woog Kang[1], Ji Yun Jeong[3], Jong Gwang Kim[1] and Yee Soo Chae[2]

[1]Kyungpook National University Medical Centre, Daegu, Republic of Korea; [2]Kyungpook National University Hospital, Daegu, Republic of Korea; [3]Soonchunhyang University Gumi Hospital, Soonchunhyang University College of Medicine, Gumi, Republic of Korea

Purpose: We previously demonstrated a prognostic role of exosomal Del-1 with breast cancer patients. However, the mechanisms of Del-1 expression are barely understood. Development of resistance to tamoxifen is an important clinical issue in the treatment of breast cancer. Accordingly, we investigated the function of Del-1 in tamoxifen-resistant (TAM^R) breast cancer cell line.
Methods: Del-1 expression in MCF7 and TAM^R MCF7 cells was performed by quantitative RT-PCR, western blot and ELISA. The effects of

Del-1 with RNA interference on proliferation, migration and invasion of TAM^R MCF7 cells were observed by MTT, wound healing and Matrigel transwell assay.
Results: Del-1 was highly expressed in TAM^R MCF7 cells compared to MCF7 cells. Moreover, down-regulation of Del-1 inhibited the proliferation and migration of TAM^R MCF7 cells. There was no difference in the invasion of TAM^R MCF7 cells.
Conclusion: Prominent expression of Del-1 in TAM^R MCF7 cells was associated with the proliferation and migration of TAM^R MCF7 cells. Accordingly, our findings suggest that the expression of Del-1 promote tamoxifen resistance in breast cancer cells and could be a novel target for anti-breast cancer treatment.

PF01.12

Chloride intracellular channel protein 4 (CLIC4) is a serological cancer biomarker released from tumour epithelial cells via extracellular vesicles

Vanesa C. Sanchez[1], Alayna Craig-Lucas[2], Bih-Rong Wei[2], Anjali Shukla[2], Abigail Read[2], Ji Lou[2], Mark Simpson[2], Kent Hunter[2] and Stuart Yuspa[2]

[1]NIH; [2]LCBG NCI NIH

CLIC4 is a highly conserved metamorphic protein originally described as an ion channel. It translocates to the nucleus serving as an integral component of TGF-β signalling. In multiple cancers, CLIC4 is a tumour suppressor, excluded from the nucleus and lost from the cytoplasm of progressing cancer cells. In contrast, CLIC4 is upregulated in the tumour stroma in response to TGF-β. CLIC4 lacks a secretory sequence, but recent reports indicate that CLIC4 is detected in the circulation of cancer patients serving a possible biomarker and has been detected in extracellular vesicles (EVs).
EVs from cell culture supernatants or biological fluids from SKOV3/SCID xenograft ovarian and 6DT1 orthograft breast cancer models, were isolated by differential centrifugation, following ultracentrifugation and Optiprep density gradients. EV size distribution and concentration were analysed by NTA and TEM. The presence of markers and CLIC4 were analysed by immunoblot.
We validated the presence of CLIC4 in EVs released into supernatants from primary normal and multiple ovarian tumour cell lines. Substantial increases in CLIC4 were measured in EVs of tumour cells when compared to normal cells. TGF-β-induced myofibroblasts also increased CLIC4 in both the cells and the EVs they released. Immunostaining analysis of human ovarian cancer tissue arrays show CLIC4 preferentially expressed in tumour stroma of multiple subtypes with the exception of ovarian serous adenocarcinomas, where it is upregulated in both compartments. *In vivo*, CLIC4 levels increased in EVs released into the peritoneal cavity as tumour burden increased in a heterotopic xenograft ovarian cancer model. Moreover, CLIC4 levels in EVs isolated from plasma increased with tumour burden and lung metastatic load in an orthotopic syngeneic mouse breast cancer model. To dissect the contribution of stromal vs. tumour epithelial compartments as the source of the EVs, CLIC4 was deleted in breast cancer cell lines by CRISPR/Cas9. CLIC4 in circulating EVs is reduced in CLIC4 KO tumour-bearing mice when compared to WT, indicating that the major contribution of CLIC4 into circulation is from tumour epithelium.
CLIC4 levels in EVs from biological fluids may have value as a cancer biomarker, in conjunction with other markers, to detect or analyse tumour progression or recurrence.

Poster Session F02 – EV Isolation: Developments
Chairs: Charles Lai and TBD 5:15–6:30 p.m.

PF02.01

Evolution of next generation affinity-based extracellular vesicle isolation technologies for liquid biopsy and therapeutic purposes

Sébastien Fournier[1], Ian C. Chute[2], Annie-pier Beauregard[2], Catherine Taylor[1], David Barnett[2], Andrew Joy[2], Nguyet Nguyen[1], Biji Anish[1], Jeremy Roy[1], Awanit Kumar[2], Sheena Fry[2], Nicolas Crapoulet[3], Morgan Brianne Dawn Stephenson[1], Simi Chacko[2], Sami Benzina[2], Remi Richard[1], Stephen M. Lewis[4], Rodney J. Ouellette[4] and Anirban Ghosh[5]

[1]Atlantic Cancer Research Institute, New Brunswick, Canada; [2]Atlantic Cancer Research Institute, New Brunswick, Canada; [3]Department of Chemistry and Biochemistry, Faculty of Medicine, Université de Sherbrooke, Quebec, Canada; [4]Department of Chemistry and Biochemistry, Université de Moncton, New Brunswick, Canada; [5]Department of Chemistry and Biochemistry, Université de Moncton, New Brunswick, Canada

Introduction: Given the tremendous potential of circulating extracellular vesicles (EVs) for liquid biopsy and therapeutic applications, there is a great demand for simple, robust and clinically-adaptable EV isolation methods. Ultracentrifugation, ultrafiltration and antibody-based EV isolation methods provide significantly less yield compared to polymer-based EV precipitation. Currently available polymer-based EV isolation methods are toxic and non-specific, thereby hindering therapeutic and diagnostic applications. To address these challenges we have developed and validated next generation affinity-based EV capture technologies that use a synthetic peptide (Vn96) or non-toxic clinically-approved polysaccharides.
Methods: We have used electron microscopy, atomic force microscopy, nanoparticle tracking analysis, immunoblotting, cellular uptake assays, a cellular tra/nsformation assay, proteomic analysis and nucleic acid detection to analyse the EVs isolated using our affinity-capture methods.
Results: The Vn96 peptide provides an easy and efficient EV isolation method using only small bench-top centrifugation for precipitation, and is also amenable to bead-based batch purification. Similarly, hyaluronic acid and chitosan-based affinity purification of EVs were developed, validated and advanced for therapeutic isolation of EVs. We found superior efficacy of our methods for multiparametric downstream molecular analyses of nucleic acid and protein biomarkers, which enables liquid biopsy assays for limited clinical sample volume. Our technologies allow easy separation of EVs from the isolation matrices, which permits functional assays such as cellular uptake, cargo delivery and cellular transformation. These properties enable downstream manipulation of captured EVs for therapeutic applications.
Conclusion: Our results indicate that the clinical compatibility, scalability, quality, platform versatility, and cost-effectiveness of our EV isolation technologies provide multiple advantages over currently-available methods. Our development of scalable non-toxic EV isolation technologies opens new opportunities for future fundamental EV research, as well as EV-based therapeutics.

PF02.02

Plasmonic detection of extracellular vesicles in a microfluidic environment using synthetic-peptide (Vn96) based affinity capture

Srinivas Bathini[1], Duraichelvan Raju[1], Simona Badilescu[1], Rodney J. Ouellette[2], Anirban Ghosh[2] and Muthukumaran Packirisamy[1]

[1]Concordia University, Montreal, Canada; [2]Department of Chemistry and Biochemistry, Université de Moncton, New Brunswick, Canada

Introduction: Extracellular vesicles (EVs) are groups of nano-scale extracellular communication organelles which contain disease biomarkers for cancer and other pathological conditions. In this work, we have developed a novel method to detect and characterise EVs by using a label-free localised surface plasmon resonance (LSPR) method based on the sensitivity of the gold plasmon bands to the environment of gold nanoparticles.
Methods: EVs from different sources are detected and characterised by using a plasmonic platform, based on gold nanoparticles. First, a complete plasmonic sensing protocol is established and carried out by using gold nanoparticles on glass substrates and, subsequently, the procedure is transferred in a microfluidic environment. Gold nanoparticles are deposited on glass substrates by a thermal convection method and annealed to form gold nano-islands that are highly sensitive plasmonic platforms. In this protocol, EVs are affinity-captured by a polypeptide named Vn96, attached to the biotin-streptavidin couple. Gold nano-islands on glass are bonded to a 2 mm thick PDMS, containing a 200 µm wide channel with a collection chamber of 5 mm diameter. The different chemicals involved in the protocol are flown through the channel at a rate of 10 µL/min. After each step, the spectrum is measured and the shift of the Au LSPR band is determined with respect to the previous stage.
Results and Conclusion: A calibration curve showing the shift of the gold plasmon band for different concentration of EVs is plotted for different cell lines. A low detection limit of EVs is found in the case of breast cancer cell-line (MCF7) generated conditioned media grown in small bioreactor. Compared to the macro detection method, the microfluidic detection of EVs proved to be highly reproducible and more sensitive as very small amounts of chemicals and EVs are necessary for the analysis.

PF02.03

Acoustic trapping of extracellular vesicles in biological fluids

Anson T. Ku[1], Hooi Ching Lim[1], Mikael Evander[2], Hans Lilja[3], Thomas Laurell[1], Stefan Scheding[1] and Yvonne Ceder[1]

[1]Lund University, Sweden; [2]Department of Biomedical Engineering, Lund University, Sweden; [3]Memorial Sloan Kettering

The diverse role of extracellular vesicles (EVs) in physiological function such as clotting, conferral of immunity, and cell signalling have recently begun to emerge. It has been implicated that EVs in urine and plasma may contain diagnostic and prognostic information, *i.e* in cancer. Based on EVs' accessibility as a non-invasive source of biomarkers, large-scale investigations into the EV contents in clinical cohorts should be a priority. To date, a major challenge in evaluating whether molecular profiling of EVs contributes important clinical value is the lack of a rapid, efficient, low cost method for enriching EVs that are amendable to use in routine practice. Here, we demonstrate a novel automated technique to enrich EVs, termed *acoustic trapping*, based on secondary acoustic forces arising from ultrasonic waves scattering between 12 µm seeding particles and extracellular vesicles in a resonant cavity. Our data show that we can successfully enriched EVs from conditioned media from SHSY5Y neuroblastoma cell line, as well as from human-derived urine and plasma samples. Furthermore, we found that, similar to ultracentrifugation, acoustically trapped samples contained vesicles ranging from exosomes to microvesicles, as demonstrated by nanoparticle tracking analysis and transmission electron microscopy. Interestingly, we did not observe any Tamm Horsefall proteins contaminations in the urinary samples enriched by acoustic trapping that were present when using ultracentrifugation. The enriched vesicles were unaffected by ultrasonic waves as determined by TEM and yielded detectable level of miRNAs by qRT-PCR and our data indicates that that the bulk of the miRNAs are contained within the vesicles. Importantly, EV preparation were obtained starting from only 200 µL of sample volume, in less 30 min of enrichment time per sample. Thus, the time, volume, and ease-of-use factors of the acoustic trapping technology make it an ideal method for biomarker discovery and potentially future routine clinical use. Taken together, we have shown that acoustic trapping can overcome the challenges inherent in ultracentrifugation method and prove to be a fast, automated, low-volume compatible, and robust method to enrich EVs from different biological fluids.

PF02.04

Capturing EpCAM-positive extracellular vesicles by programmable bio-surface

Mitsutaka Yoshida[1], Kazuhiro Hibino[2], Sachiko Matsumura[3], Tamiko Minamisawa[3], Kazuya Iwai[1], Satoshi Yamamoto[3] and Kiyotaka Shiba[4]

[1]Tokyo Dental College, Tokyo, Japan; [2]Cancer Institute; [3]Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan; [4]The Cancer Institute of Japanese Foundation of Cancer Research, Tokyo, Japan

Introduction: Because extracellular vesicles (EVs) are released from almost all types of cell, bodily fluids contain a mixture of these EVs. If these mixtures are analysed without further differentiation, the results will represent the average features of the mixtures, which would negatively affect the precision of EV-based diagnosis.
Methods: For differentiating cancer-related EVs from other EV mixtures, a coating agent that could endow the surfaces of various inorganic materials with an affinity to EpCAM (CD326) was developed.
Results: We focused on EpCAM because it is expressed in epithelial cells and not in most blood cells, making it an ideal cancer marker for blood-based diagnosis. The agent developed is composed of peptide aptamer for EpCAM and a Zwitterionic polymer, 2-methacryloyloxyethyl phosphorylcholine (MPC). Preferential binding of EpCAM-positive EVs over negative ones to the surface of polystyrene substrate coated with the agent was confirmed through atomic force microscope observations.
Conclusion: This coating agent, EpiVeta, could be implemented with various diagnostic devices, allowing for the concentration of cancer-related EVs from EV mixtures.

PF02.05

Plasma microvesicles/exosome enrichment and purification by a block-copolymer based method

Zhenyu Zhong, Matthew Rosenow, Janet Duncan, Mark Miglarese, Kiyotaka Shiba[1], Nick Xiao and David Speztler

Caris Life Sciences

Introduction: Microvesicles (MVs)/exosomes-based liquid biopsy has recently attracted a great attention for both proteomic and cancer diagnostics interests. However, lack of fast and reliable sample handling protocol for enriching/purifying these micro particles undermines most of the studies. Current approach to enrich MV/exosome in biological fluid includes Ultra Centrifuge, PEG8000-based precipitation and affinity capture. Unable to process a small amount of sample, this tedious procedure prevents it from large-scale studies. Heterogeneity and lack of clear MVs/exosome unique markers cast great limitation in affinity-related methods. Lack of selectivity for PEG-related method results in precipitation of too much free high abundant proteins in biological fluid; it also might not be compatible with a variety of the downstream applications.
Methods: Pluronic block copolymer F68 was adopted to precipitate MV/exosomes. Various concentrations of the copolymer were tested for MV/exosome precipitation efficiency in a plasma sample with spike-in cell line exosome, the MV/exosome identity was examined by DLS, TEM, FLOW as well as ELISA and the content of the enriched MV/exosome fraction was profiled by NGS and semi-quantitative mass spectrometry. The profile was also compared to two commercially available PEG-related exosome enrichment protocols.
Results and Summary In a cell line exosome spike-in setting, it achieved closed to 100% of recovery with much less protein contaminants compared to the PEG-related methods. The isolated plasma MVs/exosome was confirmed to be enriched in exosome related protein CD9 by multiple applications (ELISA/FLOW/western blot/TEM), DLS and TEM shows the isolated MVs/exosome consistent with the exosome size range. NGS shows exosome-related microRNA, such as let-7 family. MS analysis revealed more MVs/exosome-related protein enriched compared to the PEG-based method. In summary, we have developed a MVs/exosome purification method from biological fluid that could be more convenience to apply on a larger scale study and perform multiple level of downstream analysis.

PF02.06

Fast and reproducible purification of extracellular vesicles using combined size exclusion and bind-elute chromatography

Giulia Corso[1], Imre Mäger[2], André Görgens[1,3], Matthew J. Wood[2], Joel Z. Nordin[1]and Samir EL-Andaloussi[1,2]

[1]Department of Laboratory Medicine, Karolinska Instiutet, Stockholm, Sweden; [2]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom; [3]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

Introduction: Purification is one of the biggest challenges in the field of extracellular vesicles (EVs) due to their small size and physiochemical properties. Ultracentrifugation (UC) is the current gold standard isolation method, however it has several disadvantages, recent studies indicate that due to the high forces, the vesicles aggregate, fuse and break, likewise it is operator dependant and time consuming. Here, we describe a novel chromatography method for EV purification that overcome these issues.
Methods: The commercially available chromatography column is built on an activated core bead technology and combines bind-elute with size exclusion chromatography (BE-SEC). To verify the feasibility of this method for EV purification, cell-culture supernatant from different cell sources was purified on the BE-SEC column. Isolated particles were characterised by nanoparticle tracking analysis, western blot and electron microscopy. To investigate if the BE-SEC isolation method affected the physical properties of EVs, an uptake study using flow cytometry was performed.
Results: Our data show that the BE-SEC technique isolates intact vesicles, ranging around 100 nm in size with a classical EV shape. Common EV markers were present, whereas Golgi and ER contaminants were not detected. Additionally, the BE-SEC samples were depleted of non-vesicular proteins and RNAs according to SEC fractionation. When compared to UC isolated EVs, the purity was higher in the BE-SEC purified samples and the recovery yield was exceeding 70%. Moreover, UC and BE-SEC isolated EVs exhibited the same surface proteins and were equally taken up in recipient cells irrespective of the purification method used.
Conclusion: In this study, we show that the BE-SEC method can be used for EV purification from small to large amounts of cell-conditioned media, achieving high-yield and pure EVs in a time-efficient manner. Furthermore, the method does not affect EVs physical properties and surface protein signature.

PF02.07

On-chip liquid biopsy: progress in isolation of exosomes for early diagnosis of cancer

Navneet Dogra[1,2], Carlos Cordon-Cardo[2], Jungreem Woo[2], Gustavo Stolovitzky[1,2]

[1]IBM; [2]Icahn School of Medicine, NY, USA

In contrast to a standard biopsy, the so-called "liquid biopsy" offers a rapid, non-invasive, and cost effective alternative for cancer diagnosis. Exosomes, which are vesicles secreted by most eukaryotic cells and range in size from 30–150 nm, are the target biomarkers in this technique as they carry a diverse variety of genetically rich cargo, including proteins, RNA and DNA. Additionally, the size and quantity of exosomes correlate with cancer and other diseases. Hence, studying exosomes could potentially provide vital information about undesirable genetic deviations occurring in their cell of origin. Rapid isolation of exosomes from blood, urine or other body fluids remains a key challenge in this growing field.
Deterministic lateral displacement (DLD) pillar arrays have proven an effective means to sort, segregate, and enrich micron-size particles, such

as parasites and blood cells. Here, we have developed a nanoscale DLD device, containing gap sizes as small as ~25 nm, with nanoscale sorting resolution of biological particles. This development in nano-fluidics and engineering has enabled us to sort colloidal particles at the tens of nanometres scale. Additionally, we have developed predictive computational models to provide key insights into the behaviour of particles in these systems. Furthermore, we have successfully demonstrated on-chip, size-based separation of exosomes, indicating the potential of this technology for sorting plasma, urine, serum or circulating tumour-derived exosomes.

PF02.08

Withdrawn by author

PF02.09

Identification and characterisation of single-chain Fv antibodies specific to CD9 for high efficient recovery of exosomal vesicles

Yoichi Kumada[1], Ryota Akai[1], Aranna Nemoto[1], Kazutaka Matoba[2], Junko Katayama[2] and Jun-ichi Horiuchi[1]

[1]Kyoto Institute of Technology, Kyoto, Japan; [2]Nissan Chemical Industries Ltd

Introduction: Efficient, precise and inexpensive separation technologies for exosomal vesicles are one of attractive topics, and they must be essential for next-generation of miRNA-based clinical diagnosis. Here, we demonstrated use of single-chain Fv (scFv) antibodies as a ligand protein for specific separation of exosomal vesicles from culture supernatant as well as human serum. ScFv antibodies are recombinant fusion proteins of antibody fragments that VH and VL domains of monoclonal antibody were connected via a flexible peptide linker $(G_4S)_3$. Isolation and identification of scFvs specific to target biomolecules can be achieved by conventional phage display technology, while characterisation of isolated scFvs, such as production level, binding affinity, specificity and remaining activity in immobilisation state would be significantly important for industrial use. Here, we reported identification and characterisation of anti-CD9 scFv antibodies for immunoaffinity separation of exosomal vesicles.

Methods: Rabbit spleen immunised with 293T cells overexpressing native CD9 was used for preparation of scFv-displayed phage library. According to the original biopanning procedure, five candidates of scFvs were identified from the library. Antigen-binding affinity and specificity of these scFvs were characterised by BIAcore, flow cytometry and western blotting.

Results and Conclusion: EC2-hFc that extracellular domain II of CD9 was genetically-fused with Fc fragment of human IgG was used as a model antigen. Furthermore, their binding to antigen on cell membrane of Hela cells was successfully confirmed by flow cytometer. Site-specific immobilisation of scFv to PS latex beads could be achieved via our original material-binding peptides and consequently, anti-CD9 scFv was stably immobilised with high density and remaining activity. It was revealed that the exosomal vesicles released from Hela cells were selectively separated by our original scFv-immobilised beads, according to the results of flow cytometry and western blotting. Thus, the scFv antibodies identified by our original method will be significantly useful for comprehensive and specific separation of exosomal vesicles.

Poster Session F03 – Bodyfluid Biomarkers of Cancer
Chairs: TBD and Maja Puhka 5:15–6:30 p.m.

PF03.01

Identification of non-invasive prostate cancer biomarkers by miRNA deep sequencing analysis of urinary extracellular vesicles

Marta Rodriguez-Moreno[1], Cristina Bajo-Santos[2,3], Viktor Berge[4], Aija Linē[2] and Alicia Llorente[1]

[1]Oslo University Hospital-The Norwegian Radium Hospital, Oslo, Norway; [2]Latvian Biomedical Research and Study Centre; [3]University of Latvia, Riga, Latvia; [4]Department of Urology, Oslo University Hospital, Oslo, Norway

Please see OPT01.03

PF03.02

Development and testing of EV- and prostate cancer specific monoclonal antibodies

Maija Puhka[1], Maarit Takatalo[2], Teijo Pellinen[1], Olli Kallioniemi[1], Antti Rannikko[3], Marjo Yliperttula[4], Elina Serkkola[5], Saara Laitinen[6], Pia R-M. Siljander[2], Taija af Hällström[1,5], Laura-Leena Kiiskinen[7] and Sari Tiitinen[7]

[1]Institute for Molecular Medicine Finland FIMM, University of Helsinki, Helsinki, Finland; [2]Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland; [3]Helsinki University Central Hospital, Department of Urology, Helsinki, Finland; [4]Division of Pharmaceutical Biosciences and Centre for Drug Research, Faculty of Pharmacy, University of Helsinki; [5]Orion Corporation, Orion Pharma, Espoo, Finland; [6]Finnish Red Cross Blood Service, Helsinki, Finland; [7]Medix Biochemica, Espoo, Finland

Introduction: Extracellular vesicle (EV) research field needs analytical tools to support the booming basic research and quest for better biomarkers. We developed monoclonal antibodies (Mabs) against urinary EVs derived from patients with aggressive prostate cancer (Pca) and characterised their binding to EVs from Pca patients and various other sources.
Methods: Small and large EVs were isolated with differential centrifugation from pooled urine samples derived from 12 Pca patients (Gleason score 8–9) and used to immunise mice. The produced Mabs were screened with our low-input ELISA-test for binding to Pca (Gleason score 6–9, and post-prostatectomy) or control EVs from various sources as well as to common contaminant proteins THP, BSA and PSA. Mabs were further characterised for their binding to EVs or EV proteins (CD9 and CD63) by ELISA, quantitative immuno-EM, Apogee flow cytometry and western blotting. Immunohistochemistry (IHC) was used to visualise staining of different cancer and control tissues on tissue microarrays (TMAs).
Results: Antibody titers indicated successful immunisation with both EV types. ELISA screen of Mabs starting from >3000 clones revealed nine clones that produced antibodies binding preferentially to Pca EVs, urinary EVs, small or large EVs or many types of EVs. Out of the nine Mabs, one showed preferential binding to the urinary EVs from Pca patients relative to controls in ELISA, immuno-EM and Apogee flow cytometry, but was not functional with the tested protocols in IHC or western blotting. The other eight Mabs were also tested with these techniques, which mostly confirmed the binding specificities detected by the initial ELISA testing. With three Mabs, IHC revealed in most cases enriched staining to the luminal side on the epithelium as expected from a secretory target. However, the tested Mabs didn't show any clear cancer specific staining pattern. None of the nine Mabs recognised CD9 or CD63.
Conclusion: We have successfully produced and characterised novel EV-specific Mabs, with one antibody showing potential for Pca detection in urine samples and several others for ubiqutous or source-dependent recognition of EVs. These Mabs can be used as novel tools in EV research and diagnostics.

PF03.03

Purification and characterisation of plasma-derived EVs for early cancer diagnosis

Eline Oeyen[1], Hanny Willems[1], Geert Baggerman[1], Gerhard Weber[2], Kurt Van Mol[3], Patrick Pauwels[4] and Inge Mertens[5]

[1]University of Antwerp/VITO, Antwerp, Belgium; [2]FFE Service; [3]Pharmafluidics; [4]UZA; [5]University of Antwerp, Belgium

Introduction: Liquid biopsies offer great potentiel in cancer diagnostics because they contain EVs that are secreted directly by the tumour. To exploit this potential, the biggest challenge is the purification and characterisation of these EVs, in order to start from pure samples in proteomics-based biomarker discovery experiments
Methods: In this study we use plasma samples (approved by the Ethics committee of the University of Antwerp) to optimise purification procedures as a first step in proteomics-based biomarker discovery. To evaluate all used methods for purification (size exclusion chromatography (SEC) and free-flow electrophoresis (FFE)), we used assymetrical-flow field-flow fractionation coupled with UV and multi-angle light scattering detectors (AF4/UV-MALS), western blot, NTA and TEM. AF4 can also be used a s a separation technique and will also be evaluated for purification. Protein content of the obtained fractions was identified using LC-MS/MS on the Q-exactive mass spectrometer.
Results: Using SEC separation of plasma, we are able to enrich EVs in certain fractions, as shown by Western blot, TEM and NTA, but SEC is not sufficient to separate EVs completely from platelets and bulk proteins, mainly lipoproteins. When we combine SEC and AF4, the UV-MALS output looks promising, but because of the large dilution factor during SEC and AF4 separation, downstream applications are not yet possible. Standalone AF4/UV-MALS and FFE are still under evaluation for platelet free plasma. Preliminary results show great potential for both techniques.
Conclusion: Both FFE and AF4/UV-MALS show great potential for the purification and characteriation of EVs, but both techniques need to be optimised.

PF03.04

Plasma extracellular vesicles as source of biomarkers for treatment response of patients with head and neck squamous cell carcinoma

Dorival Mendes Rodrigues[1], Soon Sim Tan[2], Sai Kiang Lim[2], Andre Lopes Carvalho[3], N. Gopalakrishna Yier[4] and Andre Luiz Vettore[1]

[1]Universidade Federal de São Paulo, Argentina; [2]A*STAR; [3]Hospital do Câncer de Barretos; [4]National Cancer Centre of Singapore, Singapore

Induction chemotherapy (IC) with cisplatin and paclitaxel followed by chemoradiotherapy (CRT) based on cisplatin is safe, well tolerated and improves overall survival for patients with locally advanced head and neck squamous cell carcinoma (HNSCC) (stage III–IV). However, 30% of the patients with locally advanced HNSCC treated with CRT have incomplete response to the treatment and there is no biomarker able to prospectively segregate these patients from those who respond to the treatment. Extracellular vesicles (EVs) carrying proteins and nucleic acids in the plasma may be an alternative novel source for discovery of new specific markers for patients with HNSCC. In this study, Cholera toxin B chain (CTB) and Annexin V (AV) which respectively binds GM1 ganglioside and phosphatidylserine, were used to isolate EVs from pooled plasma samples from 6 HNSCC patients who responded (R) and 6 patients who presented incomplete response (IR) to IC and CRT treatment. The isolated EVs were screened by antibody arrays to examine the protein cargo. 327 proteins were identified in CTB-EVs, of which 54 and 19 proteins were presented exclusively in R and IR patients, respectively. Further, 271 proteins were identified in AV-EVs, 20 of them were present only into R patients while other 20 proteins were restricted to IR patients. This study provides a list of 113 specific

markers that may contribute to the development of new tools for prediction and assessment of IC and CRT response and demonstrated that CTB and AV could be used to isolate circulating plasma EVs for biomarker discovery in HNSCC.

PF03.05

Metabolomics analysis of urinary exosomes reveals novel candidate biomarkers of prostate cancer

Patricia Zuniga[1], Pilar Sanchez-Mosquera[1], Ana R Cortazar[1], Esperanza Gonzalez[1], Cristina Alonso[2], Miriam Perez-Comenzaba[2], Ana Loizaga-Iriarte[3], Aitziber Ugalde-Olano[3], Isabel Lacasa[3], Azucena Castro[2], Miguel Unda[3], Arkaitz Carracedo[1] and Juan M. Falcón-Pérez[1]

[1]CIC bioGUNE; [2]OWL Metabolomics; [3]Hospital Basurto, Bizkaia, Spain

Introduction: Urine constitutes an ideal source of biomarkers, particularly for diseases of the genitourinary system. However, direct analysis of urine can be very inefficient due to low abundance of some molecules and to the low sensitivity of some techniques. Apart from soluble molecules, urine samples contain EVs that protect of degradation and in concentrate many molecules that are present in urine. Thus, isolation and characterisation of urinary EV could increase the efficiency of biomarker discovery. Our team has previously identified some proteins and RNAs that are differentially expressed in urinary exosomes from prostate cancer compared to benign prostate hyperplasia. In the current work we focused on the analysis of the metabolites contained in urinary EVs.
Methods: Urine samples were collected from patients with prostate cancer (n = 31) and benign prostate hyperplasia (n = 14), and after clearing at low centrifugation and ultrafiltration steps, EVs were isolated by differential ultracentrifugation. Afterwards, targeted metabolomics analysis of EVs was performed by ultra-high performance liquid chromatography–mass spectrometry. Correlations between metabolites and clinical parameters were statistically studied, and differentially expressed metabolites were detected and mapped into cellular pathways.
Results: In urinary EVs we have detected 300 metabolites belonging to different chemical nature including amino acids, vitamins, as well as different lipid species. We found some metabolites that significantly correlate with current markers of prostate cancer (e.g. PSA). In addition, we have detected 90 metabolites that are significantly different between prostate cancer and benign prostate hyperplasia. Apart of being candidate biomarkers for prostate cancer, the mapping of these metabolites in cellular pathways indicates that prostate cancer could altered amino acids and sterol metabolisms in cells emitting EVs
Conclusion: In this work we demonstrated that urinary EVs contain metabolites of different chemical nature. Importantly, we show that the analysis of EVs in combination with high-sensitive metabolomics approach could provide candidate biomarkers and insight in the molecular mechanism of the disease.

PF03.06

Analysis of extracellular vesicles from plasma of advanced (IIIc or IV stages) melanoma patients during kinase inhibitor and/or immunotherapy treatments

Pascal Colosetti[1], Henri Montaudié[2], Philippe Bahadoran[2], Robert Ballotti[3], Sophie Rome[4] and Corine Bertolotto[3]

[1]UMR Inserm U1060/INRA 1397; [2]Hospital; [3]INSERM; [4]INRA

Introduction: Extracellular vesicles (EVs) shaping tumour microenvironment, contribute to *pre-metastatic niche* formation, favour tumour dissemination and mediate resistance to treatments. We have previously shown that senescent melanoma cells, in response to treatment with chemotherapeutic drugs, release soluble (e.g. chemokine CCL2) and insoluble factors. The origin and roles of these insoluble factors remain to be elucidated but it is admitted that the amount of circulating EVs is a factor of poor prognosis in melanoma. Moreover, increasing line of evidences indicate that the composition of these vesicles exhibits tissue specificity. In this study we propose a pilot clinical study on patients with advanced non-resectable or metastatic melanoma to determine the effect of targeted therapies (BRAF and/or MEK inhibitors, immunotherapy) on the production (quantity and size) of EVs isolated from blood.
Methods: All patients gave written consent and the protocol was approved by ethic committee. Plasmas were collected on EDTA tubes from patients (n = 8) before treatment and 3 times during therapies. EVs were isolated by using qEV original size exclusion columns (iZON). Protein concentration (Bradford assay) and acetylcholine esterase activity (AChE) were determined on each fraction and only the fraction 8 containing the vesicles was selected. EVs were characterised by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM).
Results: Analyses of the four EV samples per patient indicated that, whatever the treatment considered, the number of plasma EVs increased during the treatment (non-significant tendency). Concomitantly, we observed a significant decrease in mean diameter of EVs before and after treatment (46.4 ± 8.3 nm *vs* 33.6 ± 6.4 nm, p = 0.011).
Conclusion: These data indicate that EV size reduction might be a marker of chemo-resistance in patients suffering from melanoma, thus suggesting their possible use as theranostic tool in their management.

PF03.07

Isolation and characterisation of urinary exosomes

Eline Oeyen[1], Geert Baggerman[2], Hanny Willems[2] and Inge Mertens[3]

[1]University of Antwerp/VITO, Antwerp, Belgium; [2]University of Antwerp /VITO, Antwerp, Belgium; [3]University of Antwerp, Belgium

Introduction: Exosomes are nanosized extracellular vesicles that are secreted by normal, diseased and tumour cells in all body fluids (i.e. plasma, breast milk and urine). Since their origin, molecular content and function, exosomes are suitable as a source of diagnostic biomarkers. In this way, urine exosomes provide a targeted view into the urogenital tract to enhance the ability to detect urological diseases or tumours and their progression.
Methods: Isolation of exosomes from urine was optimised to obtain a pure exosome fraction. Size exclusion chromatography (SEC) combined with a preprocessing step (ultrafiltration or a commercial kit) was used. The urine sample collection was approved by the Ethics committee of the University of Antwerp, comply with the Declaration of Helsinki. Isolated extracellular vesicles were characterised by techniques such as nanoparticle tracking analysis, western blotting, electron microscopy and assymetrical-flow field-flow fractionation coupled with UV and multi-angle light scattering detectors (AF4/UV-MALS). Isolated vesicles were used in down stream proteomic analysis.
Results: Western blot data demonstrated the presence of EV-specific proteins Flotillin-1, CD9, CD63 and CD81 in the EV-relevant fractions of both isolation methods. NTA results and electron microscopy images showed a higher enrichment of EVs using ultrafiltration and SEC. Proteomic analysis also demonstrated that this isolation method gives more identifications of EV-relevant proteins. The results of AF4/UV-MALS demonstrated that fraction 9 after SEC was most enriched in EVs. The size of the isolated vesicles ranged from 40 to 160 nm.
Conclusion: In conclusion, ultrafiltration combined with SEC is preferred as isolation method of EVs from urine. AF4/UV-MALS proved to be a good quality control method for urinary EVs to determine their purity and size.

PF03.08

Diagnostic and prognostic potential of miRNA alterations in blood based extracellular vesicles from clear cell renal cell carcinoma patients

Joana Heinzelmann, Diana Kuhn, Sophie Baumgart, Sebastian Hoelters, Michael Stoeckle and Kerstin Junker

Department of Urology and Paediatric Urology, Saarland University, Saarbrucken, Germany

Introduction: In previous studies we identified 14 specific miRNA alterations in tumour tissues of clear cell renal cell cancer (ccRCC) with prognostic value relating to the presence of metastasis. We hypothesise that in a simple blood based test tumour cell related

miRNA alterations can be proven in EV as biomarkers for diagnosis and evaluation of the metastatic risk.
Methods: EV were isolated from 1 ml serum of 20 ccRCC patients (6 metastatic and 9 non-metastatic tumours) and 10 healthy volunteers using differential centrifugation and EV precipitation with *exosome isolation kit* (Fisher Scientific). By nanotracking analysis (NTA) and western blot we proofed the EV concentration and quality of isolation. EV-totalRNA was isolated using *miRNeasy Mini Kit* (Qiagen). Concentration of 14 miRNAs (miR-10b, -30a-3p/5p, -30c-5p/2-3p, -30e-3p/5p, -126-3p/5p, -139-5p, -144, -204, -451 and -455-3p) was revealed by qPCR. To this, 10 ng totalRNA was reverse transcribed (TaqMan Reverse Transcription Kit, Fisher Scientific) and preamplified (TaqMan PreAmp Master Mix, Fisher Scientific). Amplification was performed using *Gene Expression master mix* (Fisher Scientific).
Results: CcRCC serum samples are characterised by threefold increased EV concentration compared to non-malignant controls. In five out of 20 serum samples, miRNA expression was too low for qPCR analyses. In the remaining 15 serum samples, two miRNAs (miR-30-2-3p and -455-3p) were not detectable. Three out of 14 miRNAs (miR-10b, -126 and -451) analysed in this proof of principle study exhibited a significantly decreased expression in serum EV compared to the controls ($p < 0.05$). But, patients with metastatic ccRCC showed no significant different miRNA expression compared to non-metastatic counterparts.
Conclusion: These initial data confirm that the tissue based miRNA signature could be used as biomarkers for detection of ccRCC analysing EV from liquid biopsies. The identified miRNAs can be used as possible markers for early detection and monitoring of metastatic disease. To validate these results the expansion of the sample set is ongoing.

PF03.09

The content of circulating exosomes changes according to malignancy of prostate cancer and trigger phenotypical changes that may promote cancer progression and metastasis

Eliana Andahur[1], Mei Yieng Chin[2], Juan Fulla[1], Alejandro Mercado[1], Christian Ramos[1], Kim Chi[2], Emma Guns[2] and Catherine A. Sánchez[1]

[1]Clinica las Condes, Santiago, Chile; [2]Vancouver Prostate Centre, Vancouver, Canada

Introduction: Cancer cells release exosomes as a mechanism of cell communication. Exosomes carry DNA, RNA and proteins that can transform the surrounding cells, promoting their growth and progression. Exosomes also reach the distant sites to prepare the pre-metastasis niche. Therefore, understanding the content of the circulating exosomes and their effects on normal cells are critical to evaluate their use as biomarkers for prostate cancer (PCa).
Methods: Blood from patients with and without PCa was collected. All patients signed the informed consent forms. Exosomes were extracted from plasma by Exoquick, then quantified by NanoSight. Total RNA and protein content were measured by Nanodrop and BCA assay, respectively. A panel of seven microRNAs was evaluated by TaqMan assay. Finally, normal epithelial cells (RWPE-1) were treated with the patients' plasma exosomes, for 96 h to assess the cell viability using MTS assay and 48 h to evaluate the expression of E-cadherin by immunocytochemistry. Exosome uptake by RPWE-1 cells was also evaluated by confocal microscopy.
Results: Exosomes were isolated from plasma from individuals without cancer (n = 10), PCa patients with Gleason score 6 ($n = 10$), ≥7 ($n = 10$) and castration-resistant disease (CRPC) ($n = 5$). We found that the total amount of plasma exosomes was similar between the four groups of sample. However, the protein and RNA content, and the levels of the seven microRNAs analysed were higher in exosomes extracted from plasma of cancer patients. In the other hand, RWPE1 cells that had uptaken PCa patient-derived exosomes showed an increased cell proliferation and diminished expression of E-cadherin when compared to those treated with exosomes extracted from normal individuals. Furthermore, such effect was greater in RWPE-1 cells treated with CRPC patient-derived exosomes.
Conclusion: In this study, we showed that the amount of circulating exosomes does not seem to differ between normal individuals and PCa patients. However, their protein content and the 7 microRNA analysed are increased in PCa patients compared to those without cancer, and thus could be an interesting source for biomarkers. In addition, we showed that exosomes isolated from PCa patients can trigger phenotypical changes on normal prostate cells, and thus may promote cancer progression and metastasis.

PF03.10

Diagnosis of prostate cancer using serum PSA and Del-1 positive exosomes in plasma

Chan-Hyeong Lee[1], Eun-Ju Im[1] and Moon-Chang Baek[2]

[1]Department of Molecular Medicine, School of Medicine, Kyungpook National University, Daegu, Republic of Korea; [2]Kyungpook National University, Daegu, Republic of Korea

Introduction: Despite the prostate-specific antigen (PSA) test is the most important screening method for prostate cancer, there is an increasing demand for biomarkers for diagnosis of prostate cancer because of high false-positive rate that lead to unnecessary prostate biopsies and over-diagnosis. Developmental endothelial locus-1 (Del-1) is an extracellular membrane protein of exosomes and commonly upregulated in multiple types of human cancers. In this study, we focused on development of new test using Del-1 positive exosomes for prostate cancer diagnosis.
Methods: Del-1 positive exosomes were measured in plasma of prostate cancer patients and healthy controls by sandwich ELISA.
Results: Del-1 positive exosomes of prostate cancer patients were significantly elevated compared with healthy controls. In addition, level of Del-1 positive exosomes was not correlated with serum PSA. Del-1 positive exosomes were increased in low PSA level prostate cancer patients.
Conclusion: Those data support that combination of Del-1 and PSA can improve sensitivity and specificity of prostate cancer screening.

PF03.11

Characterisation of small RNA content in urinary and plasma EVs and matching prostate cancer tissues

Cristina Bajo-Santos[1], Vita Melne[2], Kristīne Sobolevska[3], Pawel Zayakin[4], Arturs Ābols[4], Vilnis Lietuvietis[5], Alicia Llorente[6] and Aija Linē[4]

[1]Latvian Biomedical Research and Study Centre/University of Latvia, Riga, Latvia; [2]Riga Stradiņš University/Rīgas Austrumu klīniskā universitātes slimnica, Riga, Latvia; [3]Latvian University, Riga, Latvia; [4]Latvian Biomedical Research and Study Centre, Riga, Latvia; [5]Rīgas Austrumu klīniskā universitātes slimnica, Riga, Latvia; [6]Oslo University Hospital-The Norwegian Radium Hospital, Oslo, Norway

Introduction: It has been estimated that approximately one in seven adult men will develop prostate cancer (PCa) in the course of their life. Despite of the current diagnostic methods, there is still an unmet need to find specific biomarkers that could identify PCa in a non-invasive manner. The aim of this study was to characterise the small RNA cargo in EVs isolated from different biofluids (plasma and urine) before and after radical prostatectomy, and to compare it with the small RNA content in the matching tumour and normal prostate tissue.
Methods: Urinary and plasma derived EVs were isolated by size exclusion chromatography. EVs were visualised by electron microscopy, quantified by Nanosight and characterised by western blot analysis. Prior isolation of RNA, EVs were treated with proteinase K and RNAse A. RNA quantity and quality was assessed using Agilent Bioanalyser. Small RNA libraries were constructed from EVs and matching tumour and normal prostate tissues, and libraries were sequenced using Ion Proton platform.
Results: The isolated vesicles were positive for CD9, CD81, CD63 and Alix and negative for GM130. A total of 2.4–4.6 million reads were obtained by deep sequencing of the small RNA libraries (RNAseq). RNAseq data analysis revealed that both, urinary and plasma EVs, contained various classes of RNAs. Specifically, long non-coding (lncRNA), mitochondrial (MtRNAs), ribosomal, small nuclear, small nucleolar, micro (miRNAs), Y, vault, miscellaneous and protein coding RNAs were detected. No substantial variation in the representation of the different RNA species has been identified among biofluids, except for MtRNAs, which have been characterised mainly in plasma EVs. Interestingly, an increase in lncRNAs was detected in EVs obtained from plasma after prostatectomy. A total

number of 292 different mature miRNAs were identified. Among those, 17 miRNAs that were overexpressed in the tumour tissues were detectable in the urinary EVs, 4 in the plasma EVs and 6 miRNAs were found both, in urinary and plasma EVs, and may represent PCa biomarker candidates that warrant further validation.
Conclusion: Both, urinary and plasma EVs contain small RNAs that may be derived from the tumour tissues, yet the specific RNA signatures are substantially different between the biofluids.

PF03.12

Exosomal Del-1 as a potent diagnostic marker for breast cancer: a prospective cohort study

Soo jung Lee[1], Ho Yong Park[2], Byung Woog Kang[1], Jong Gwang Kim[1], Jeeyeon Lee[1], Jin Hyang Jung[2], Ji Yun Jeong[3], Pyong-Gon Moon[4], Moon-Chang Baek[4], Jae-hwan Jeong[1] and Yee Soo Chae[2]

[1]Kyungpook National University Medical Centre, Daegu, Republic of Korea; [2]Kyungpook National University Hospital, Daegu, Republic of Korea; [3]Soonchunhyang University Gumi Hospital, Soonchunhyang University College of Medicine, Gumi, Daegu, Republic of Korea; [4]Kyungpook National University, Daegu, Republic of Korea

Introduction: We previously demonstrated a diagnostic role of exosomal del-1 with two separated groups of breast cancer patients. In the current study, therefore, we aimed to confirm the diagnostic role in a prospective study with breast cancer by measuring plasma exosomal del-1 before and after surgery.
Methods: To identify the optimal time of sampling after surgery, serial blood at day 1, 3, 5 and 7 after surgery was collected from 22 patients with early breast cancer. Thereafter, 115 patients with breast cancer who underwent curative surgery were enrolled in the prospective cohort study to compare difference in plasma exosomal del-1 measured by ELISA at the time of diagnosis and post-surgery.
Results: Among all 22 patients for optimal sampling time after surgery, exosomal del-1 was higher than 0.5 at the time of diagnosis and then normalised at POD1. Among 115 patients for the confirmatory set, 109 (94.8%) patients showed a normalisation of del-1 lower than 0.5 after surgery and 10 patients showed del-1 >0.4. For median follow-up duration of 22 months, 9 patients experienced relapse (4 locoregional and 5 distant), with 3 out of 6 in high group (>0.5), 2 out of 4 in borderline group (0.4–0.5) and 4 out of 105 in normalised group.
Conclusion: In a prospective cohort study, we confirmed that exosomal del-1 has a potent diagnostic role in breast cancer. Furthermore, del-1 was also identified to dramatically decrease after curative surgery. Our current findings suggest its potential prognostic role as well as diagnostic role in breast cancer patients.

Poster Session F04 – EVs in the Tumour Microenvironment
Chairs: Jason Webber and TBD 5:15–6:30 p.m.

PF04.01

Extracellular vesicles derived from cancer-associated fibroblasts may have a role in oral cancer invasion

Mauricio R. Dourado[1], Johanna Korvala[2], Raija Sormunen[3], Ilkka Miinalainen[4], Sami Yokoo[5], Pirjo Åström[2], Adriana Franco Paes Leme[5], Ricardo Della Coletta[1] and Tuula Salo[6]

[1]Department of Oral Diagnosis, Piracicaba Dental School, Unicamp; [2]Cancer and Translational Medicine Research Centre, University of Oulu, Oulu, Finland; [3]Biocenter Oulu, University of Oulu, Oulu, Finland; [4]Biocenter Microscopy Service, University of Oulu, Oulu, Finland ; [5]Mass Spectrometry Facility, LNBio-CNPEM; [6]Medical Research Centre, University of Oulu, Oulu, Finland

Please see OPT01.02

PF04.02

Oral cancer EVs contain miRNA capable of promoting protumourigenic fibroblast activation

Mark Ofield, Daniel Lambert and Stuart Hunt

University of Sheffield, United Kingdom

Introduction: Oral cancer mortality rates have increased by 10% in the last decade. Efforts to reverse this are hampered by a limited understanding of the underlying molecular complexity of the disease. Recently, interest has grown in the contribution of extracellular vesicles (EVs) to cancer pathogenesis. Developing tumours consist of multiple cell types including fibroblasts, however, these bear little resemblance to their normal counterparts, but have a myofibroblast-like, protumorigenic phenotype. This project aims to evaluate the impact of EVs from oral cancer cells on normal oral fibroblasts (NOFs).
Methods: EVs were isolated from the culture media of dysplastic and carcinoma cell lines for characterisation by western blotting, TEM and TRPS. The miRNA contents of EVs were determined by next-generation sequencing. EVs were transferred to NOFs and their uptake visualised by fluorescence microscopy. The impact of this uptake on NOF proliferation (BrdU ELISA), viability (live/dead staining) and activation (western blot and immunofluorescence microscopy of α-SMA protein levels) was assayed.
Results: Oral cancer cells produced between 1500–4000 EVs/cell/24 h ranging in size from 50–200 nm and bearing the EV marker CD63. Kegg pathway analysis identified several miRNA present in EVs that target members of the TGF-β signalling pathway and are known to modulate activation of fibroblasts. EVs were readily taken up by NOFs with no significant impact on viability or proliferation. However, analysis of α-SMA protein levels showed that EV uptake was sufficient to activate NOFs to a myofibroblast-like phenotype.
Conclusion: Our data indicates that oral cancer cell-derived EVs are able to transfer miRNA to NOFs causing an increase in α-SMA expression and the adoption of a myofibroblast-like phenotype which would favour tumour progression.

PF04.03

Immunotherapy in multiple myeloma using alfa-galactosylceramide loaded sEVs from dendritic cells to stimulate NKT activity

Sylvia Faict[1], Mérédis Favreau[1], Elke De Bruyne[1], Kim De Veirman[1], Ken Maes[1], Karin Vanderkerken[1], Rik Schots[2] and Eline Menu[1]

[1]Haematology and Immunology Lab, Vrije Universiteit Brussel, Brussels, Belgium; [2]Department of Clinical Haematology, UZ Brussel, Brussels, Belgium

Introduction: Multiple myeloma (MM) is associated with an increase in immune suppression. Our lab has focused on the role of invariant natural killer T (iNKTs) in this immune suppression. A decline in iNKT cell activity was previously demonstrated, leading to a defective immune response to the myeloma cells. NKTs can be activated by alfa-galactosylceramide (aGC) when presented by dendritic cells (DCs).
Stimulation by these DCs induce a strong Th1 response, however clinical trials have been disappointing. One of the reasons is that aGC stimulation leads to such strong iNKT activation that they rapidly become anergic, hindering repeated stimulation.
Here we try to overcome this anergy by using small extracellular vesicles (sEVs) secreted by DCs loaded with aGC. sEVs derived from DCs contain the CD1d molecule, and may, in this way, also present aGC to iNKT cells, resulting in their activation.
Methods: For these experiments we use the 5T33MM model which is immunogenic and represents the human disease closely. DCs were derived from the bone marrow of naive mice and cultured for seven days before adding aGC. sEVs were isolated from the conditioned medium of 60 million DCs. The presence of CD1d positive sEVs was confirmed by Western Blot and TEM. *In vitro* properties were assessed by co-culturing the isolated sEVs with DCs and/or 2C12 cells (an iNKT hybridoma line), and measuring the IL-2 response. Unfortunately, aGC-loaded sEVs induced no clear activation of the iNKTs. Nevertheless, when injected intravenously, these sEVs did elicit an IFNy response. When 5T33MM mice were treated again seven days later, the aGC loaded sEVs produced a lasting response in contrast to the aGC loaded DCs which resulted in iNKT anergy. This sustained iNKT response translated into an anti-tumour effect in the 5T33MM mice.
Conclusion: sEVs derived from DCs can be loaded with aGC and induce an IFNy response from iNKT cells without inducing anergy *in vivo*, resulting in a reduced MM tumour load.

PF04.04

Exosomes derived from gastric cancer cells activate NF-κB pathway in macrophages to promote cancer progression

Xu Zhang and Wenrong Xu

Jiangsu University, Jiangsu, China

Exosomes are nano-sized membrane vesicles secreted by both normal and cancer cells. Emerging evidence indicates that cancer cells derived exosomes contribute to cancer progression through the modulation of tumour microenvironment. However, the effects of exosomes derived from gastric cancer cells on macrophages are not well understood. In this study, we investigated the biological role of gastric cancer cells derived exosomes in the activation of macrophages. We demonstrated that gastric cancer cells derived exosomes activated macrophages to express increased levels of proinflammatory factors, which in turn promoted tumour cell proliferation and migration. In addition, gastric cancer cells derived exosomes remarkably upregulated the phosphorylation of NF-κB in macrophages. Inhibiting the activation of NF-κB reversed the upregulation of proinflammatory factors in macrophages and blocked their promoting effects on gastric cancer cells. Moreover, we found that gastric cancer cells derived exosomes could also activate macrophages from human peripheral blood monocytes through the activation of NF-κB. In conclusion, our results suggest that gastric cancer cells derived exosomes stimulate the activation of NF-κB pathway in macrophages to promote cancer progression, which provides a potential therapeutic approach for gastric cancer by interfering with the interaction between exosomes and macrophages in tumour microenvironment.

PF04.05

TGF-β1-silenced leukaemia cell-derived exosome-targeted dendritic cells induce stronger anti-leukaemic immunity

Siguo Hao, Fang Huang and Jiangbo Wan

Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China

Tumour-derived exosomes, which could induce a specific antitumor immune response, have been developed as a promising tumour vaccine. However, the efficiency of exosomes-based vaccines in clinical trials has been unsatisfactory. In this study, we investigated whether DC pulsed with TGF-β1-silenced leukaemia cell-derived exosome ($LEX_{TGF-\beta1si}$) is more immunogenic than DC pulsed with non-modified leukaemia cell-derived exosome (LEX). We used a lentiviral vector containing TGF-β1 small hairpin RNA (shRNA) to obtain $LEX_{TGF-\beta1si}$. The prepared $LEX_{TGF-\beta1si}$ facilitated the maturation of dendritic cells (DCs) more effectively. Moreover, DCs which pulsed with LEX □$DC_{LEX-TGF-\beta1si}$) promoted more efficiently $CD4^+$ T cell proliferation and Th1 cytokine secretion. In addition, $DC_{LEX-TGF-\beta1si}$ induced a more potent tumour-specific $CD8^+$ CTL response *in vitro*. Besides, we conducted an animal study indicating that $DC_{LEX-TGF-\beta1si}$ significantly inhibited the tumour growth and prolonged the survival time in tumour-preventive and tumour-protective models. Taken together, our findings revealed that $DC_{LEX-TGF-\beta1si}$ induced specific antitumor immunity effectively, suggesting that the utilisation of $DC_{LEX-TGF-\beta1si}$ might be a promising approach to optimised TEX-based tumour vaccines

PF04.06

Phenotyping and quantification of cascade-primed immune cells (CAPRI) and their EVs

Evo K. L. Soendergaard, Rikke Baek, Malene M. Jorgensen, Kim Varming and Lotte H. Pugholm

Department of Clinical Immunology, Aalborg University Hospital, Aalborg, Denmark

Introduction: Immunotherapies used for cancer treatment are based on knowledge about the immune cells and their interactions with tumour cells. However, the exact cellular function of each individual immune cell subtype in relation to cancer cells are an ongoing investigation and could be highly influenced by extracellular vesicles (EVs). EVs have earlier been suggested to play a part in the progression of pathological conditions such as cancer and have shown to be involved in a variety of important physiological and immunological processes. EVs are one of several tools cells use to communicate with each other. The communication is facilitated by a number of surface-associated proteins and the cargo of the vesicles. The aim of this project was to phenotypically characterise the cascade-primed immune cell (CAPRI) culture used for immunotherapy (1) and their corresponding EVs and compare them to peripheral blood mononuclear cells and their corresponding EVs from five healthy blood donors.
Methods: The cells from five healthy blood donors were cultured either as peripheral blood mononuclear cells or as CAPRI cells. The cells and the cell culture supernatants were harvested at several different time points. The cellular phenotype were analysed by flow cytometry while the EVs were phenotyped (for more than 20 EV markers) and semi-quantified (CD9, CD63 and/or CD81 positive) using the EV Array (JEV) (2).
Results: Based on the flow cytometric analysis, it can be concluded that there is a general change in the composition of T cell subtypes when peripheral blood mononuclear are cultured as CAPRI cells. Moreover, it was observed that the amount of T cells was enhanced in these cultures. Overall, the cellular phenotype show similarities between individuals whereas the EV phenotypes seem to be more person-to-person affected even though similarities can be drawn.
Conclusion: These data show a potential for learning more about the cellular and vesicular communication in the immune system.

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PF04.07

Ovarian tumour cells suppress antitumor immune response through the release of arginase-1-containing exosomes

Malgorzata Czystowska-Kuzmicz[1], Marta Szajnik[1,2], Kavita Ramji[1], Dominika Nowis[1,3,4], Slawomir Gruca[1], Artur Stefanowicz[5] and Jakub Golab[1]

[1]Department of Immunology, Centre of Biostructure Research, Medical University of Warsaw, Poland; [2]Department of Gynaecology and Gynaecologic Oncology, Military Institute of Medicine, Warsaw, Poland; [3]Genomic Medicine, Medical University of Warsaw, Poland; [4]Laboratory of Experimental Medicine Centre of New Technologies University of Warsaw, Poland; [5]Department of Gynecology and Obstetrics, "Praski" Hospital, Warsaw, Poland

Introduction: Arginase-1 (Arg-1) is a cytosolic enzyme catalysing degradation of the semi-essential amino acid L-arginine. Abundant Arg-1 has been detected in either tumour cells or in tumour-infiltrating myeloid cells and correlates with depletion of L-arginine and consequent suppression of antitumor immunity. Here we report that OvCa cells release Arg-1 in tumour-derived exosomes (TEX) and investigate the influence of TEX-derived Arg-1 on the antitumor effector mechanisms of immune response.
Methods: TEX were isolated by ultracentrifugation or exclusion chromatography and verified by Western blotting, NanoSight and electron microscopy. The presence and activity of Arg-1 in TEX was determined by Western blotting and arginase activity assay. Immunohistochemical Arg-1 expression in primary OvCa were correlated to clinico-pathological characteristics. Effects of exosomal Arg-1 on immune cells were analysed by *in vitro* proliferation assay and flow cytometry.
Results: Enzymatically active Arg-1 was detected in TEX derived from patients' ascites as well as from ovarian cancer cell lines. OvCa ascites contained higher levels of exosomal Arg-1 compared to fluids obtained from benign ovarian cysts. High Arg-1 expression in primary lesions correlated negatively with intratumoral T-cell infiltrates and CD3-zeta expression and was associated with shorter time to recurrence (TTR). *In vitro*, OvCa-derived Arg-1-positive TEX (Arg1-TEX) inhibited CD8+ and CD4+ T-cell proliferation and decreased T-cell receptor expression. Co-culture of bone-marrow-derived dendritic cells (DC) with Arg1-TEX resulted in the transfer of functionally active Arg-1 and inhibition of DCs-primed proliferation of OVA-antigen specific OT-I T cells. All these *in vitro* effects were reversed by a novel Arg-1 inhibitor.
Conclusion: Our findings provide the first evidence for the role of Arg-1 in the formation of an immunosuppressive microenvironment in OvCa. We identify a novel mechanism of exosomal Arg-1 distribution from the tumour cells to antigen presenting cells. Inhibition of Arg-1 activity may be an attractive novel anti-cancer strategy.

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PF04.08

Natural killer extracellular vesicles: a functionally relevant and measurable surrogate of the natural killer activity in cancer patients

Veronica Huber[1], Cristina Federici[2], Elisabetta Iessi[2], Serena Cecchetti[2], Simona Ferro[1], Agata Cova[1] and Luana Lugini[2]

[1]Fondazione IRCCS Istituto Nazionale dei Tumori; [2]Istituto Superiore di Sanità

Introduction: Natural killer (NK) cells belong to the innate immunity, represent the first-line defence in the control of tumour growth and are key players in immunosurveillance. Defective NK activity is associated with and increased risk to develop cancer. NK cells release extracellular vesicles (EVs) endowed with cytotoxic activity against tumour cells. Their anti-tumour effects appeared to be mediated by a surface-to-

surface interaction and also by internalisation of EVs by the tumour cells. The killer molecules carried by NK EVs included FasL and perforin. NK EVs, detectable in plasma, could thus represent a functionally relevant and measurable surrogate of NK activity in cancer patients.
Methods: We developed an ad hoc exosome-immune enzymatic test (NKExoELISA) to study the phenotype of plasmatic NK EVs. This test measures the expression of exosome markers concomitantly with typical NK markers and results were confirmed by Western blot and flow cytometry analysis. NK EVs, isolated from NK cell conditioned media, were also immunoassayed by Cytometric Bead Array. The functionality of identified molecules was evaluated by tests of cell death induction, proliferation and activation in flow cytometry.
Results: NKExoELISA can discriminate and measure NK EVs, identified as exosomes, among the vesicles present in human plasma of both healthy donors and cancer patients, based on their concomitant expression of tsg-101/CD9 and CD56/NKG2D. Apart from FasL and perforin, NK EVs carry TRAIL, IFN gamma, IL-2 and marked amounts of granzyme B. The expression of CD62L suggests that NK EVs possess the potential to home to sites of injury and inflammation, such as cancer. The cytotoxic potential, measured by AnnexinV and propidium iodide, correlated with concentration of FasL and granzyme B carried by EVs. Co-culture of NK EVs with PBMCs from healthy donors induced rosette-forming cells, typical signs of proliferation.
Conclusion: Our results suggest that NK EVs may represent a measurable surrogate of NK cell activity in plasma. NK EVs exhibit a rich equipment of killer molecules and appear to possess immunostimulating activities. This could be potentially exploited to revive the anergic status of anti-tumour immunity, commonly observed in cancer patients.

PF04.09

Heparan sulphate proteoglycans as regulators of exosome-induced stromal cell differentiation

Alexandra Shephard[1], Zsuzsanna Tabi[1], Aled Clayton[2] and Jason P. Webber[1]

[1]Cardiff University, Cardiff, United Kingdom; [2]Division of Cancer and Genetics, School of Medicine, Cardiff University and Velindre Cancer Centre, Cardiff, United Kingdom

Introduction: We have shown that prostate cancer exosomes trigger fibroblast differentiation to a disease-supporting myofibroblast phenotype, and have implicated heparan sulfate proteoglycans (HSPGs) in this process. Here we characterise HSPGs present on prostate cancer exosomes and further explore the role of exosomal-HSPGs in regulation of fibroblast differentiation.
Methods: A lentiviral-based shRNA approach was used to selectively knockdown HSPGs in prostate cancer cells. Exosomes were isolated by flotation and characterised by nanoparticle tracking, western blot and plate-based assays. Fibroblasts were stimulated with exosomes, and differentiation to a myofibroblast phenotype was determined by the onset of α-smooth muscle actin (αSMA).
Results: Exosomes from different prostate cancer cell lines express variable levels of TGFb, which correlate with expression of HSPGs on the exosome surface. TGFb-high exosomes express syndecan 3, syndecan 4, glypican 1, glypican 6 and betaglycan. We have generated prostate cancer cell lines that secrete exosomes lacking specific HSPGs. These HSPG-deficient exosomes show a reduced ability to drive fibroblast differentiation.
Conclusion: Exosomal, not soluble, delivery of TGFβ is essential for generating a disease-like stroma. This exosome function is dependent on HSPGs, such as betaglycan, present on the exosome surface. Exosomal-HSPGs may therefore represent novel targets for attenuating tumour growth.

PF04.10

Exosomes derived from mesenchymal stem cells promotes bone regeneration in hyperhomocysteinemia mice

Jyotirmaya Behera, Yuankun Zhai, Akash K. George, Suresh C. Tyagi and Neetu Tyagi

University of Louisville, KY, USA

Introduction: It is critical that bone formation and angiogenesis are tightly coordinated during bone regeneration, but the molecular regulator of such intercellular communication in bone microenvironment is not well studied. Cystathionine-β-synthase (CBS), an enzyme in homocysteine metabolic pathway, plays an important role in osteoblast differentiation. Therefore, we hypothesise that CBS as a novel molecular regulator in mesenchymal stem cell derived osteoblasts, which regulates vascularisation during bone development in osteoblast derived exosomes.
Methods: To test this hypothesis, we used 12 weeks old male mice in our study, including wild type mice (C57BL/6, WT) and CBS± mice. Bone regeneration was evaluated by microCT and angiogenesis assay.
Results: Blocking the CBS function by either inhibitor hydroxylamine (HA) prevented osteoblast differentiation and mineralisation. This was supported by studies using osteoblasts cultured from bone marrow of CBS deficient (CBS±) mice. Exosomes of mesenchymal stem cell derived osteoblasts cultured stimulated endothelial migration and angiogenesis, which was prevented by blocking CBS in osteoblasts using HA. Mass spectrometry analysis and ELISA assay identified among others vascular endothelial growth factor (VEGF)-A and VEGF homologue placental growth factor (PIGF) to be present in CBS(+/+) mice osteoblasts but not in CBS± osteoblasts. Metatarsal angiogenesis assay showed retarded vascularisation in bone tissue of CBS± mice. CBS± mice showed significantly reduced bone mineral density (BMD) and bone volume/tissue volume (BV/TV) by microCT and showed increased plasma homocysteine levels compared to CBC+/+ mice (WT). Furthermore, real-time PCR and western blot analyses revealed significant decreases Alkaline phosphatase (ALP) and Runt-related transcription factor 2 (RUNX2) and Osteocalcin (OCN) expression in CBS-deficient (CBS±) mouse bone marrow cells.
Conclusion: We demonstrate that CBS in mesenchymal stem cell derived osteoblasts is at the crossroad of osteoblast differentiation/mineralisation and angiogenesis. These findings uncover previously undefined molecular understanding of CBS that promote angiogenesis and osteogenesis in bone development and regeneration.

PF04.11

Exosomes from mutant KRAS colorectal cancer cells reprogram the metabolic state of recipient cells

Qin Zhang, Dennis Jeppesen, James Higginbotham, Henry Manning, Jeffrey Franklin and Robert Coffey

Vanderbilt University Medical Center, TN, USA

Mutant KRAS colorectal cancer (CRC) cells exhibit increased aerobic glycolysis with elevated levels of the glucose transporter SLC2A1 (hereafter GLUT1). Whether mutant KRAS cells alter the metabolic state of the tumour microenvironment is unknown. Herein, we show mutant KRAS CRC cells (DLD-1 and DK0-1), compared to their isogenically matched wild-type KRAS counterparts (DKs-8), release exosomes containing increased functional GLUT1 as determined by ^{18}F-fluorodeoxyglucose (FDG) uptake. Exosomes released from GLUT1 knockdown DLD-1 cells exhibit dramatically reduced FDG uptake, demonstrating that GLUT1 is the major glucose transporter in these cells. In addition, we show that mutant KRAS-derived exosomes induce cellular metabolic changes in recipient cells, including enhanced glucose consumption and increased glycolysis, as determined by an increased NADH to FAD ratio. Systemic delivery of mutant KRAS exosomes also enhances glutamate/cystine exchange in $Apc^{Min/+}$ colonic tumours, using a novel PET tracer, ^{18}F-FSPG. Thus, CRC cells with activating KRAS mutations may alter the metabolic state of recipient cells via exosomes containing high levels of GLUT1, a process that may nourish the tumour microenvironment and fuel tumour progression.

PF04.12

Extracellular vesicles released following heat stress induce bystander effects in unstressed populations

Findlay R. Bewicke-Copley[1], Laura A. Mulcahy[2], Laura A. Jacobs[3], Priya Samuels[1], Ryan C. Pink[1] and David R.F. Carter[1]

[1]Oxford Brookes University, Oxford, United Kingdom; [2]Ashfield Healthcare Communications; [3]Technical University of Munich, Munich, Germany

Introduction: The bystander effect is a phenomenon where the effects of stress occur in naïve cells through signalling from nearby stressed cells. We previously showed that bystander effects induced by ionising radiation are mediated by extracellular vesicles (EVs). Bystander effect can also be induced by other types of stress, including heat shock, but it is unclear whether EVs are involved.
Methods: Cells were heat shocked at 45°C and 24 h later EVs were extracted from the cell culture medium using ultracentrifugation. These EVs were then used to treat cells naïve to the stress conditions. Cells were incubated with EVs for a further 24 h before being assayed for DNA damage, Apoptosis and Cell viability using the Comet assay, nuclear fragmentation assay and MTT assay respectively.
Results: Here we show that EVs released from heat shocked cells are also able to induce bystander damage in un-stressed populations. Naïve cells treated with media conditioned by heat shocked cells showed higher levels of DNA damage and apoptosis than cells treated with media from control cells. Treating naïve cells with EVs derived from media conditioned by heat shocked cells also induced a bystander effect when compared to control, with DNA damage and apoptosis increasing whilst the level of cell viability was reduced. We demonstrate that treatment of naïve cells with heat shocked cell-derived EVs leads to greater invasiveness in a trans-well matrigel assay. Finally, we show that naïve cells treated with EVs from heat-shocked cells are more likely to survive a subsequent heat shock, suggesting that these EVs mediate an adaptive response.
Conclusion: We propose that heat shock causes the release of a subpopulation of EVs from cells that leads to apparent stress in neighbouring cells but also greater robustness in the face of a subsequent insult.

PF04.13

Galectin-3 binding protein present at the surface of tumour exosomes contributes to their capture by stromal cells

Rie Nakata[1], Laurence Sarte[1], Pascale Zimmermann[2] and Yves A. DeClerck[3]

[1]Children's Hospital Los Angeles, CA, USA; [2]University of Marseille, Marseille, France; [3]University of Southern USA

Introduction: Galectin-3 binding protein (Gal-3BP/*LGALS3BP aka*: MAC2-binding protein) is a 90 kDa secreted sialoglycoprotein that is commonly present in the cargo of exosomes and is among the 25 common cancer proteins associated with extracellular vesicles (EVs) secretion in all NCI-60 cancer cell lines (1). Here we have examined its presence and function in exosomes from human neuroblastoma cells that we had previously reported to secrete Gal-3BP (2).
Methods: The expression of Gal-3BP was examined in exosomes from 10 human NB cell lines by western blot analysis. Exosomes were prepared by differential ultracentrifugation (DUC), Optiprep density gradient centrifugation (ODGC) and size exclusion chromatography (SEC). Gal-3BP localisation in cells and exosomes was performed by confocal microscopy, flow cytometry and electron microscopy. Its role in exosome biogenesis and capture by stromal cells was examined in NB cells in which the *LGAL3SBP* gene was removed by CRISPR-Cas9 knock out.
Results: Gal-3BP was consistently present in all preparations of exosomes obtained from 10 NB cell lines. It was also present in exosomes from the plasma of patients with NB. It was consistently associated with exosome protein markers like CD-63, syntenin and ALIX in exosomes obtained by DUC, ODGC and SEC, in addition to being present in a soluble form in the culture medium of NB cells. However in NB cells Gal-3BP was clearly segregated from CD-63, suggesting its absence in mulitivesicular bodies and an absence of involvement in exosome biogenesis. This was further supported by the demonstration that syntenin knock down in NB cells did not affect the presence of Gal-3BP in exosomes. We then demonstrated by a combination of flow cytometry and enzymatic digestion, that Gal-3BP is present on the surface of exosomes. To better understand its function, *LGALS3BP* was knocked out in NB cells. Whereas Gal-3BP KO did not affect the production of exosomes in NB cells, it inhibited their capture by stroma cells.
Conclusion: Our data bring insight into the function of a protein commonly identified in the cargo of cancer cell exosomes, suggesting an absence of involvement in exosome biogenesis and a role in exosome uptake by stromal cells.

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Poster Session F05 – Inflammatory Disorders, Tissue Injury and Coagulation
Chairs: TBD and Riend Nieuwland 5:15–6:30 p.m.

PF05.01

Human podocyte microparticles impair proliferation of proximal tubule epithelial cells

Suzy Sun[1], Mercedes Munkonda[1], Eldjonai Kamto[1], Christopher Kennedy[1] and Dylan Burger[2]

[1]Kidney Research Centre, Ottawa Hospital Research Institute, Ottawa, Canada; [2]Kidney Research Centre, Ottawa Hospital Research Institute, University of Ottawa, Canada

Introduction: Tubular injury is a major pathogenic component of advanced chronic kidney disease. Repair following tubular injury is heavily dependent on proximal tubule epithelial cell (PTEC) proliferation. Our lab recently reported formation of microparticles (MPs) from glomerular epithelial cells (podocytes) and release into the urine in animal and human diabetes. In the present study we examined the effect of podocyte MPs on PTEC proliferation and examined the role of cell cycle inhibitory proteins and tumour suppressors in this process.
Methods: MPs were isolated from media of human podocyte (hPod) cultures by differential centrifugation. PTECs were treated with 10 μg/mL of isolated hPod MPs for 24 hrs. Proliferation was quantified by cell counting, and BrdU incorporation. Proteins associated with cell cycle arrest (p21, p27, p53), and DNA replication (proliferating cell nuclear antigen, PCNA) were analysed by western blot.
Results: After 24 hrs, PTECs treated with hPod MPs exhibited significantly lower cell viability compared to untreated PTECs (~73% reduction, $p = 0.0054$). A BrdU proliferation assay showed a 69% reduction in absorbance of BrdU in MP-treated PTECs
Conclusion: MPs isolated from hPods inhibit PTEC proliferation, however the mechanism of action requires further investigation. Nevertheless our data suggest that podocyte MPs may contribute to kidney injury by inhibiting the tubular repair process.

PF05.02

Carbon dioxide-induced oxidative stress: microparticle production and inflammasome activation by neutrophils are linked

Stephen R. Thom, Veena Bhopale and Ming Yang

University of Maryland School of Medicine, MD, USA

Introduction: We hypothesised that elevations of carbon dioxide (CO_2) often found in modern buildings will stimulate leukocytes to produce microparticles (MPs, 0.1–1 μm diameter, annexin V-positive) and activate the nucleotide-binding domain-like receptor 3 (NLRP3) inflammasome due to mitochondrial oxidative stress.
Methods: Human and murine leukocytes were exposed *ex vivo* in buffer equilibrated with air plus CO_2 at 0.1–1% and the buffer and cells separated for flow cytometer MPs analysis and biochemical assays.
Results: Neutrophils, but not monocytes, generate MPs with high interleukin-1β (IL-1β) content after 30 or more minutes exposure to air + 0.1–0.4% CO_2. Enhanced MPs production requires mitochondrial reactive oxygen species production (assessed with MitoSOX red), mediated by activities of pyruvate carboxylase and phosphoenolpyruvate carboxykinase (verified with small inhibitory RNA knock-down). Events leading to MPs generation include perturbation of inositol 1,3,5-triphosphate receptors, a transient elevation of intracellular calcium, activation of protein kinase C and NADPH oxidase. Concomitant activation of type-2 nitric oxide synthase yields secondary oxidants resulting in actin S-nitrosylation and enhanced filamentous actin turnover. Proteins are linked to short filamentous actin, including vasodilator-stimulated phosphoprotein, focal adhesion kinase, the phospholipid translocation enzymes flippase and floppase, and the critical inflammasome protein Apoptosis-associated Speck protein with CARD domain (ASC). Oligomerisation of the inflammasome components ASC, NLRP3, caspase 1, thioredoxin interacting protein and calreticulin lead to IL-1β synthesis.
Summary: An increased production rate of MPs containing elevated amounts of IL-1β persists for hours after short-term CO_2 exposures. Mean CO_2 levels in many modern buildings exceed 0.1%. The so-called "sick building syndrome" is without clear pathophysiology and MPs ladened with IL-1β may play a role.

PF05.03

Exosomal miR-185-5p modulates ADAMTS13 transcription in liver fibrosis

Qinqin Xiang, Yuanyuan Fu, Yi Ma, Fen Xu, Wen Chen and Zhou Zhou

State Key Laboratory of Cardiovascular Disease, Beijing Key Laboratory for Molecular Diagnostics of Cardiovascular Diseases, Diagnostic Laboratory Service, Fuwai Hospital, National Centre for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Metalloproteinase ADAMTS13, which is mainly derived from hepatic stellate cells (HSCs), specifically cleaves multimeric VWF to regulate thrombi formation. Recent studies have shown that miRNAs actively regulated the transcription of ADAMTS family proteins. Exosomes, a small vesicle known to mediate intercellular communication by delivering nucleic acids and proteins, draws our attention because of its ability in packaging miRNAs and protecting them from degradation. To further understand the underlying mechanisms of ADAMTS13 production and provide insights into the molecular mechanisms of coagulation function disorder in liver fibrosis, we focused on the regulation of ADAMTS13 transcription by exosomal miRNAs in both liver fibrotic mouse model and primarily cultured HSCs. To induce liver fibrosis, CCL4 was delivered intraperitoneally into male Swiss Webster three times per week for 5 weeks, with Olive oil used as vehicle control. Primary HSCs were isolated by in situ enzymatic dissociation and density gradient centrifugation from livers of Swiss Webster male mice. Candidate miRNA miR-185-5p was predicted to bind ADAMTS13 3′-UTR to regulate its transcription based on microRNA.org database. We observed that ADAMTS13 levels was down-regulated while miR-185-5p was up-regulated in the livers fibrosis mouse model or in activated primary mouse HSC, which further confirmed that miR-185-5p was associated with the down-regulation of ADAMTS13. ISH, luciferase assay and mimic, inhibitor transfection experiment demonstrated that miR-185-5p can target ADAMTS13 3′-UTR to regulate its transcription. The exosomes was isolated from medium or peripheral blood using ExoQuick kit, which were bi-membrane vesicles, 50–150 nm nanoparticles under transmission electron microscopy, and positive for flotilin and CD9. miR-185-5p was present in both mouse plasma and HSC exosomes. We observed that miR-185-5p was up-regulated in the exosomes derived from plasma of fibrosis mouse model. The transfer of Exosomes transfected with miR-185-5p mimics to quiescent HSC lead to the down-regulation of ADAMTS13 in target cells. Accordingly, exosomal transfer of miR-185-5p may be a paradigm for the regulation of ADAMTS13 transcription.

PF05.04

Effects of human and porcine mesenchymal stem cell-derived conditioned media on coagulation and T-cell function

Arezoo Mohammadipoor[1], Ben Antebi[1,2], Teryn R. Roberts[1], Kerfoot P. Walker[1], Robbie K. Montgomery[1], Andriy I. Batchinsky[1,3] and Leopoldo C. Cancio[1]

[1]United States Army Institute of Surgical Research, TX, USA; [2]ZCore Business Solution; [3]The Geneva Foundation, WA, USA

Introduction: Systemic administration of mesenchymal stem cells (MSCs) is associated with several potential health risks. MSCs have been shown to protect injured tissue, in part, by secretion of a large

variety of bioactive factors and extracellular vesicles (EVs); thus, cell-free products from MSCs are becoming more attractive candidates. In cell culture, these mediators are found in conditioned media (CM). We hypothesised that CM are safe for clinical application by evaluating the thrombogenicity and immunomodulatory potential of CM *in vitro*.
Methods: To obtain CM, human and porcine bone marrow-derived MSCs were incubated with serum-free medium. After 24 h, supernatant was collected and cells were removed by centrifugation. Thrombogenicity of CM was tested by thromboelastography (TEG). Whole blood from healthy human and porcine donors was mixed with CM at different ratios (CM: blood ratios of 1:1, 1:2.5, 1:5, 1:10, $n \geq 3$). To study the immunomodulatory effect of CM, mononuclear cells (MNCs) derived from healthy donors were labelled with a proliferation dye and stimulated to induce T-cell proliferation. MNCs were then plated with MSCs or CM in triplicates. After 72 h, T-cells were collected and assessed by flow cytometry.
Results: We observed that porcine CM significantly accelerated the initiation of clot formation (R) in a dose-dependent manner. Porcine CM also increased the rate (K, α-angle) of early clot formation related to rapid fibrin accumulation. In addition, porcine CM increased the clot strength (MA). By comparison, only the highest dose of human CM (1:1) significantly reduced the R value. However, neither K, α-angle, nor MA were affected by human CM at any ratio. MSCs reduced T-cell proliferation via cell-cell contact, yet CM did not generate the same effect.
Conclusion: In this study, we developed an *in vitro* method to evaluate thrombogenicity of CM. Our results suggest that in a porcine model, but not human, a pro-coagulant effect occurs. However, further studies are required to determine if this response is repeated *in vivo*. Also, the fraction of CM, EVs or EV-free CM, responsible for this effect remains to be elucidated. While the CM did not inhibit T-cell proliferation, it remains to be seen whether the EV fraction will produce the same results.

PF05.05

Circulating exosomes attenuate hepatic stellate cell activation and are anti-fibrotic *in vivo*

Li Chen[1], Ruju Chen[1], Sherri Kemper[1] and David Brigstock[1,2]

[1]The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA; [2]Department of Surgery, The Ohio State University, Columbus, OH, USA

Introduction: Exosomes are nanovesicles produced by many cells which contain a complex molecular cargo that can be delivered to target cells to cause functional re-programming. This study investigated if hepatic stellate cells (HSC) are regulated by circulating exosomes. HSC are the principal fibrosis-producing cells of the liver, undergoing a process of activation by which they become collagen-producing αSMA-positive myofibroblasts. Fibrosis is a major pathological feature of chronic liver disease that affects individuals globally but lacks FDA-approved therapeutics.
Methods: Exosomes were purified by ultracentrifugation from the serum of healthy or fibrotic Swiss Webster male mice, or of healthy human male donors, and characterised by nanoparticle tracking analysis, TEM and western blot. The function of exosomes was tested by their effect on (i) activation in primary cultures of mouse HSC, or (ii) CCl_4-induced liver injury in mice.
Results: Isolated exosomes from mice or human sera were bi-membrane vesicles, 80–150 nm in diameter, and positive for CD81 and flotillin-1. Exosomes (10 μg/ml) from the serum of healthy mice caused decreased connective tissue growth factor (CTGF), αSMA or collagen α1(I) mRNA levels after treatment of D9 (activated) primary HSC for 24 h ($p < 0.01$), whereas gene expression was not diminished by serum exosomes from fibrotic mice. The same dose of serum exosomes from healthy human blood donors (22–27 yo) attenuated collagen expression after treatment of human LX2 HSC for 36 hrs (p4 injury model in male transgenic mice expressing GFP under the control of the CTGF promoter, liver fibrogenesis (assessed by hepatic GFP or αSMA expression) was attenuated by i.p. administration (40 μg/g q.o.d.) of serum exosomes from healthy mice, but not from fibrotic mice ($p < 0.01$). In 5-wk CCl_4 fibrosis models, i.p. administration of serum exosomes (40 μg/g q.o.d.) from healthy mice during the last 2 wks of CCl_4 treatment caused a dose-dependent decrease in hepatic GFP-CTGF production. This was associated with decreased expression of CTGF, αSMA or collagen, as well as suppressed fibrosis.
Conclusions: These studies show that circulating exosomes from healthy individuals are instrinsically anti-fibrotic and offer a new lead for therapy of liver fibrosis.

PF05.06

Interplay of RANTES chemokine and CCR5+ bearing microvesicles in diabetic retinopathy

Aleksandra Tokarz[1], Anna Elżbieta Drożdż[2], Iwona Szuścik[3] and Ewa Stępień[2]

[1]Department of Clinical Biochemistry, Jagiellonian University Medical College, Krakow, Poland; [2]Department of Medical Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University, Krakow, Poland; [3]Private Ophthalmology Practice, OKO-LASER Outpatient Clinic

Introduction: RANTES (regulated on activation, normal T-cell expressed and secreted), otherwise known as CCL5, belongs to the C-C family of chemokines, secreted by T cells, macrophages, platelets and certain types of cancer. Among different receptors, the main one is the G-protein-coupled CCR5, which was documented on membrane derived micrvesicles (MVs). In patients with diabetes mellitus (DM), it was observed that the number of mesenchymal and monocyte origin MVs is higher in those with microangiopathies. It was also observed that the number of platelet and monocyte origin MV gradually increases with the severity of non-proliferative diabetic retinopathy (NPDR) to the proliferative (PDR).
Methods: Total 61 DM patients (63 [59–68] y.e.) and 25 control subjects (50 [45–56] y.e.) were included to the study. The diagnosis and classification of retinopathy were carried out on the basis of the Polish Diabetes Association recommendations (2016). Finally, among examined DM patients 7 had soft non-proliferative diabetic retinopathy (SNPDR), 5 had moderate non-proliferative (MNPDR), 13 had heavy non-proliferative (HNPDR) and 6 had PDR. MVs profiling ($CCR5^+$) in plasma was performed by means of Gigamix (BioCytex) calibrated CytoFLEX (Beckman Coulter). This study has permission of the Bioethical Committee of Jagiellonian University (KBET/206/B/2013)
Results: RANTES concentration was significantly elevated in DM patients with compere to healthy control, in plasma and in MV fraction (15.5 [9.7–18.1] vs. 8.9 [0.9–14.6] μg/mL, $p = 0.011$ and 14.9 [8.9–17.8] vs. 6.7 [0.9–14.1] μg/mL, $p = 0.028$). Higher concentrations of RANTES MV fraction were observed in the HNPDR group ($p = 0.041$). Total number of $CCR5^+$ MV was significantly lower in DM patients with compare to control (62 (21–185) vs. 108 (49–293) n/μL, $p = 0.049$). Interestingly, HNPDR was characterised with the higher number of $CCR5^+$ MVs in compare to PDR and other forms of NPDR.
Conclusion: Correlation between NPDR retinopathy progression and the RANTES axis was proven. The increased number of $CCR5^+$ MVs observed in HNPDR patients, it suggests pro-angiogenic activity of MVs in the advanced stage of non-proliferative DR.

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PF05.07

Characterisation of microRNA-containing extracellular vesicles secreted by bronchial epithelial cells in allergic airway inflammation

Sabine Bartel[1], Jochen Behrends[1], Andrea Schamberger[2], Oliver Eickelberg[2] and Susanne Krauss-Etschmann[3]

[1]Research Centre Borstel, Leibniz Centre for Medicine and Biosciences, Member of the German Centre for Lung Research (DZL), Germany; [2]Comprehensive Pneumology Centre, Institute of Lung Biology and Disease, Member of the German Centre for Lung Research (DZL), Helmholtz Zentrum München, Germany; [3]Division of Experimental Asthma Research, Research Centre Borstel, Leibniz-Centre for Medicine and Biosciences, Germany, Member of the German Centre for Lung Research (DZL), Institute for Experimental Medicine, CAU Kiel, Germany

Background: microRNAs (miR) are critical regulators of signalling pathways and have been shown to be essential for the development both of the immune system and the lung. miRNAs have also been shown to be secreted into extracellular vesicles (EV) for inter-cell communication (1). In previous work, we identified an up-regulation of miR-17 and -21 in lung homogenate of mice upon ovalbumin (OVA) induced allergic airway inflammation (AAI).
Objective: To assess if miR-17 and -21 are secreted into EVs in murine AAI and human bronchial epithelial cells.
Methods: EVs were isolated from broncho-alveolar lavage fluid (BALF) from mice with OVA- or house dust mite (HDM) induced AAI. Primary normal human bronchial epithelial (NHBE) cells (Lonza, Switzerland) were cultured at the air-liquid interface and treated with Interleukin (IL) 13 to model a T-helper 2 environment. EVs were isolated by Exoquick-TC (System Biosciences, USA) or qEV columns (Izon Science, UK) from BALF or basal culture medium/apical surface wash of NHBE cultures and characterised by pure or bead-based flow cytometry and western blot for CD63, HLA-DR, HSP70. miRNA levels were assessed by qRT-PCR.
Results: In both murine models for AAI, miR-17 and -21 were significantly up-regulated in isolated BALF EVs, while numbers of $CD63^+$ EV were similar. In human primary NHBE cells, $CD63^+$ EVs were found in both the apical surface wash as well as the basal culture medium. and EVs from both compartments were positive for HSP70 and HLA-DR, while secretion patterns changed with IL13 treatment. After 24 h of IL13 treatment, miR-17 (transiently) and miR-21 levels were increased in the basal cell compartment and after 7 d on the apical surface.
Conclusion: miRNA-containing EVs are secreted upon IL13 treatment of primary NHBE cells and contain more miR-17 and -21 in BALF of murine AAI. Thus, we speculate that early miRNA secretion via EVs might be involved in the development of allergic airway inflammation. Currently, we are profiling miRNAs in EVs from primary NHBE cells to investigate differences in apical and basal EV populations.

Reference

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PF05.08

IgM rheumatoid factor present on circulating extracellular vesicles obtained from rheumatoid arthritis patients can result in false positive immunoassays

Onno Arntz, Bartijn Pieters, Rogier Thurlings and Fons van de Loo

Radboudumc

Purpose: Rheumatoid arthritis (RA) is a systemic disease with autoantibodies in the circulation. In this study we investigated whether autoantibodies such as rheumatoid factor (RF) interfere with the detection of cytokines on plasma extracellular vesicles (pEVs) isolated from RA patients.
Methods: pEVs were obtained from 32 RA patients and 35 healthy controls (HC) by size exclusion chromatography. Protein content was measured by micro-BCA, size and concentration by Nanoparticle Tracking. TNFα, IL-1β and IL-6 were detected by bead-based multiplex immunoassays (Luminex). TNFα was also detected by flowcytometry (FC) and to control for specificity pEVs were preincubated with anti-TNFα antibodies of a different isotype (Enbrell and Humira). IgM-RF was measured in the pEV isolates by ELISA. RF+ and RF- pEVs were preincubated with Protein L beads to bind RF-IgM before TNFα was measured by FC. PKH26 labelled pEVs bound and unbound to protein L beads were measured in a fluorometer.
Results: Total concentration of pEVs ($2.46–4.09 \times 10^{10}$/ml), particle size (122–125 nm) and protein per EV particle (71–218 fg) of HC versus RA was not statistically different. In 13 out of 32 RA patients levels of TNFα, IL-1β and IL-6 were detectable (220, 18 and 47 pg/ml, respectively) in pEVs by Luminex while in HC these cytokines were undetectable. Presence of TNFa on RA-pEVs was confirmed by FC and preincubation with anti-TNFα completely blocked the signal. Only in the cytokine positive RA-pEV samples RF-IgM was detectable. More pEVs from RF + RA patients bound to protein L beads than RF– patients (8.4 vs. 1.2%). No TNFα was detectable in pEVs by FC after preincubation with protein L beads.
Conclusion: This study shows that IgM-RF is present on pEVs isolated by SEC from RF+ RA patients. The presence of IgM-RF may lead to a false positive signal in immunoassays and flow-cytometry. We advice to measure RF in pEV samples and pretreat samples with protein-L when pEVs will be analysed in immunoassays.

PF05.09

Outer membrane vesicles from Escherichia coli can contribute to cardiac dysfunction in sepsis

Kyong-Su Park[1], Kristina Svennerholm[2], Cecilia Lässer[1], Ganesh Shelke[1], Rossella Crescitelli[1], Su Chul Jang[1], Shintaro Suzuki[1], Elga Bandeira[1], Charlotta Olofsson[3] and Jan Lötvall[1]

[1]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden; [2]Anaesthesiology and Intensive Care Medicine, Institute of Clinical Science, Sahlgrenska Academy, University of Gothenburg, Sweden; [3]Department of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Sweden

Introduction: Sepsis is commonly associated with cardiac dysfunction, which significantly worsens the prognosis seriously for patients. It is known that Gram-negative bacteria have the capacity to release outer membrane vesicles (OMVs), which are the bilayered proteolipids with nano-sized diameters. These OMVs are composed of lipopolysaccharides, outer membrane proteins, and lipids, and can be recognised by the innate immune system to induce inflammation. The aim of this study is to determine whether OMVs from sepsis patients can induce cardiac dysfunction and to elucidate the mechanism involved.
Methods: *E. coli* was collected from the blood of a patient with urosepsis. OMVs were isolated from *E. coli* cultures by ultracentrifugation. OMVs were analysed by nanoparticle tracking and transmission electron microscopy (TEM). Cell viability, reactive oxygen species (ROS), and cytokine production were evaluated for cytotoxicity and inflammation in the cardiac muscle cell (HL-1). To check contractile dysfunction, intracellular Ca^{2+} measurements were performed using dual-wavelength ratio imaging in fura-2 loaded HL-1. Mice were intraperitoneally injected with OMVs (15 μg), and then sacrificed at 6 h. Innate inflammation was assessed using quantification of cytokines in the heart lysates and OMVs proteins were detected by polyclonal anti-OMVs antibody.
Results: The OMVs were characterised by spherical bilayered shape with diameters of 25–200 nm in TEM. Nanoparticle tracking analysis showed that the ratio of the particles ($\times 10^6$) per ng of OMVs proteins was 5.3 ± 0.5. OMVs induced cell death with production of ROS, and increased slightly the pro-inflammatory cytokines *in vitro*. Moreover, HL-1 cells subjected to OMVs displayed irregular Ca^{2+} oscillations with a decreased frequency. Using a mouse model, we showed that OMVs caused a dramatic increased in the production of TNF-α and IL-6, and delivery of OMVs proteins to the heart was confirmed.
Conclusion: This study shows that septic *E. coli* OMVs induce cardiac injury *in vitro* and *in vivo*, and can be crucial a causative microbial signals in septic cardiomyopathy. The role of OMVs in clinical disease warrant further studies, as bacterial OMVs in addition to live bacteria may be good therapeutic targets to control the infectious diseases.

PF05.10

Characterisation of exosomal miRNA profiles in patients with sepsis and septic shock

Marlene Reithmair[1], Dominik Buschmann[2], Melanie Maerte[3], Benedikt Kirchner[2], Daniel Hagl[4], Ines Kaufmann[4], Alexander Chouker[5], Ortrud Steinlein[6], Michael Pfaffl[2] and Gustav Schelling[5]

[1]Institute of Human Genetics, University Hospital of Ludwig-Maximilians, University Munich, Munich, Germany; [2]Division of Animal Physiology and Immunology, TUM School of Life Sciences Weihenstephan, Technical University Munich, Germany; [3]Department of Anaesthesiology, University Hospital, Ludwig-Maximilians-University, Munich, Germany; [4]Department of Anaesthesiology, Neuperlach Hospital, City Hospitals of Munich, Germany; [5]Department of Anaesthesiology, University Hospital, Ludwig-

Maximilians-University, Munich, Germany; [6]Institute of Human Genetics, University Hospital, Ludwig-Maximilians-University Munich, Germany

Introduction. Septic shock is a medical condition with high mortality and long-term negative consequences for cognitive and psychosocial functioning. Pro- and anti-inflammatory responses of the organism are key mechanisms in this highly lethal disorder. Cell-to-cell communication within the immune system plays an important role in regulating the interaction between pathogens and the host immune system. Liquid biopsies assessing exosomal microRNA (miRNA)-profiles could represent an important means of deciphering cell-to-cell communication in sepsis-related states and allow an early diagnosis, as well as the timely identification of patients at risk for a negative outcome.
Methods. In this study, we characterised blood-derived exosomal miRNA profiles of sepsis and septic shock patients by next-generation sequencing. We aimed at identifying differentially regulated miRNAs, and detecting previously unknown sepsis-associated miRNAs. Informed consent was obtained and the study was approved by the medical ethics committee of the University Hospital Ludwig-Maximilians, University of Munich.
Results. In septic shock, a total of 24 and 34 distinct exosomal miRNAs were down- and upregulated, respectively. The majority of these differentially regulated miRNAs in exosomes ($n = 32$) had not previously been associated with sepsis. RT-qPCR experiments on 8 of these miRNAs verified all of them except for one. Interestingly, exosome analysis contributed significant information regarding disease staging and survival prediction. Three miRNAs displayed stringent correlation of expression levels and disease severity, whereas miR-30a-5p and miR-125b-5p predicted survival of sepsis patients with high confidence. *In silico* analysis highlighted crucial signalling functions of differentially regulated miRNAs in sepsis-relevant pathways including inflammation, hypoxia signalling and pathogen sensing.
Conclusion. This study established robust miRNA expression profiles in blood-derived exosomes of sepsis patients, suggesting new avenues for sepsis research, early sepsis diagnosis, disease staging and survival prediction via liquid biopsy.

PF05.11

Functional properties of lung-tissue derived extracellular vesicles in a model of asthma

Shintaro Suzuki[1], Lilit Hovhannisyan[2], Cecilia Lässer[1], Kyong-Su Park[1], Yasunari Kishino[1], Ganesh Shelke[1], Rossella Crescitelli[1] and Jan Lötvall[1]

[1]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden; [2]Institute of Molecular Biology, Armenian National Academy of Sciences, Yerevan, Armenia

Introductions: Asthma is a chronic airway disease associated with eosinophilic inflammation. Immune cells such as Th2 lymphocytes play an important role to aggravate the inflammation by producing multiple pro-inflammatory cytokines. Multiple Th2 cytokines, including IL-13, are known to be involved in allergic asthma, and influence airway hyperresponsiveness and remodelling. Extracellular vesicles such as exosomes and microvesicles are present in the lungs, but little is known concerning their biological function in asthma. This study aims is to determine the effects of EVs on T-cell migration and cytokine release.
Material and methods: Male C57 BL/6 mice were sensitised and exposed to ovalbumin (OVA). EVs were isolated from lung tissue. EVs were visualised by electron microscopy and characterised by western blotting, and particle numbers were acquired by nano-tracking analysis (ZETA view®). CD4+ T lymphocytes were separated from the spleen of using magnetic separation. Migration of CD4+ T lymphocytes was performed using a Boyden chamber assay. The supernatant of cultured CD4+ T lymphocytes were collected 6 days after re-stimulation of OVA and treatment with EVs and used for the analysis of cytokine release by ELISA.
Results: Asthmatic lung EVs dose-dependently induced migration of CD4+ spleen T lymphocytes (numbers of migrated cells, 1040, 1057, 1525, 2673 (concentration of exosomes – 0%, 0.1%, 1%, 10%), 791, 883, 1354, 1680 (concentration of microvesicles – 0%, 0.1%, 1%, 10%)). EVs also increased IL-13 release by CD4+ cells, and microvesicles induced greater cytokine release compared to exosomes, which seemed to be suppressive (1523 pg/ml (control), 3676 pg/ml (exosome), 7357 pg/ml (microvesicles), 3780 pg/ml (OVA re-stimulation + exosome), 9410 pg/ml (OVA re-stimulation + microvesicles)).
Conclusion: Lung tissue derived EVs regulate T-cell migration and Th2 cytokine release. EVs from asthmatic lung may aggravate inflammation further, but the role of exosomes and microvesicles may be different.

PF05.12

Altered miRNA expression in neutrophil derived-exosomes in severe asthma

Amandine Vargas and Jean-Pierre Lavoie

Université de Montreal, Montreal, Canada

Asthma affects over 235 million individuals worldwide. Repeated exposures to environmental antigens cause persistent inflammation and damage to the lungs in asthma, leading to a progressive loss of airway function and a decreased life quality. There is a clear association between neutrophilic airway inflammation and severe asthma, but their causal relationship remains largely unexplored. Neutrophils are known to release a variety of mediators that promote the induction and recruitment of other immune cells, and we have recently demonstrated that they secrete exosomes abled to interact with airways smooth muscle (ASM) cells, increasing proliferation. Exosomes are enriched in miRNA fragments, which could influence the mRNA activities in recipient cells and promoting different biological processes. Using TaqMan™ microRNA assays, we investigated the expression of 12 miRNAs capable of regulating ASM fate (miR-21, miR-146a, miR-26a, miR-133a, miR-145, miR-Let7 family, miR-25, miR-143, miR-221, miR-199, mir155 and miR-214), in neutrophils derived-exosomes from severe asthmatic horses ($n = 6$) and age-matched controls ($n = 6$). These animals spontaneously developed a condition sharing marked similarities with human neutrophilic asthma. All selected miRNAs were detected in exosomal extracts, but only miR-21 was differentially expressed, with a decreased expression in exosomes from asthmatic animals compared to controls. Equine ASM cells were then transfected with miR-21 (or a miR-negative control), stimulated with LPS (100 ng/ml) for 24 h and the mRNA expression of PDCD4, IL-8 and IL-10 was measured using qPCR. Survival was also analysis using a coulter counter. Our preliminary results indicated that miR-21 increases ASM cell viability without altering the expression of the genes we studied. In conclusion, neutrophil exosomes carry several miRNAs possibly implicating in the ASM biology in asthma. MiR-21 related gene expression requires further investigations.

PF05.13

Withdrawn at request of author.

Poster Session 06 – EVs and Stem Cells I
Chairs: Bernd Giebel and Sai-Kim Lim 5:15–6:30 p.m.

PF06.01

Mesenchymal stem cell-derived extracellular vesicles alter differentiation competence of fibroblasts

Motohiro Komaki[1], Masayuki Tooi[2], Naoki Yokoyama[3], Hirohito Ayame[3], Kengo Iwasaki[1], Yuichi Izumi[2] and Ikuo Morita[4]

[1]Department of Nanomedicine, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Tokyo, Japan; [2]Department of Periodontology, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Tokyo, Japan; [3]Life Science Laboratory, Research and Development Centre, Dai Nippon Printing Co., Ltd.; [4]Department of Cellular Physiological Chemistry, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Tokyo, Japan

Introduction: The therapeutic potential of mesenchymal stem cells (MSCs) may be attributed partly to humoral factors and extracellular vesicles (EVs). Human term placental tissue-derived MSCs (PlaMSCs), or conditioned medium of these cells, have been reported to enhance wound healing. Recently, the EVs, which can transport a diverse suite of macromolecules, has gained attention as a novel intercellular communication tool. However, the potential role of the EVs in PlaMSC therapeutic action is not well understood. The purpose of this study was to evaluate whether PlaMSC-derived EVs modulate differentiation competence of fibroblasts *in vitro*.
Methods: MSCs were isolated from human term placental tissue by enzymatic digestion. Conditioned medium was collected after 48-h incubation in serum-free medium (PlaMSC-CM). EVs were prepared by ultracentrifugation of PlaMSC-CM, and confirmed by transmission electron microscopy (TEM), dynamic light scattering (DLS), and western blot analyses. The expression of stemness-related genes, such as OCT4 and NANOG, in normal adult human dermal fibroblasts (NHDF) after incubation with PlaMSC-exo was measured by real-time reverse transcriptase PCR analysis (real-time RT-PCR). The effect of PlaMSC-exo on OCT4 transcription activity was assessed using Oct4-EGFP reporter mice-derived dermal fibroblasts. The stimulating effects of PlaMSC-exo on osteoblastic and adipocyte-differentiation of NHDF were evaluated by alkaline phosphatase (ALP), and Alizarin red S- and oil red O-staining, respectively. The expression of osteoblast- and adipocyte-related genes was also assessed by real-time RT-PCR
Results and Conclusion: The treatment of NHDF with PlaMSC-exo significantly upregulated OCT4 and NANOG mRNA expression. PlaMSC-exo also enhanced OCT4 transcription. The NHDF treated with PlaMSC-exo exhibited osteoblastic and adipocyte-differentiation in osteogenic and adipogenic induction media. PlaMSC-exo increase the expression of OCT4 and NANOG mRNA in fibroblasts. As a result, PlaMSC-exo influence the differentiation competence of fibroblasts to both osteoblastic and adipocyte-differentiation. It shows a new feature of MSCs and the possibility of clinical application of MSC-exo.

PF06.02

Stimulation of adipose tissue-derived mesenchymal stem cells by monocyte- and osteoclast-derived extracellular vesicles

Arjen Gebraad[1], Sippy Kaur[1], Yusuf Khan[2], Suvi Haimi[1], Riitta Seppänen-Kaijansinkko[1] and Bettina Mannerström[1]

[1]Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, Finland; [2]Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland

Introduction: In recent years, extracellular vesicles (EVs) have gained interest as a biomimetic tool to induce lineage-specific differentiation of stem cells. Osteoclasts are the bone-resorbing cells that are formed by fusion of monocytes. Like monocytes, osteoclasts provide pro-osteogenic signals to mesenchymal stem cells (MSCs). The role of EVs in these pro-osteogenic signals is currently unknown. In this study, we performed a genome-wide transcriptome analysis of the pro-osteogenic potential of osteoclast-derived EVs in human adipose tissue-derived MSCs (AT-MSCs).
Methods: Human monocytes were isolated from buffy coats by gradient centrifugation and immunomagnetic selection. The monocytes were either activated by lipopolysaccharide or stimulated to generate osteoclasts using M-CSF and RANK-L on culture plastic or coatings of hydroxyapatite. Hydroxyapatite mimics the mineral component of bone. EVs were isolated from the conditioned medium of these cultures using a commercial precipitation kit. Human AT-MSCs were cultured for 18 days in control medium supplemented with EVs from the monocyte- and osteoclast cultures. AT-MSCs cultured in control medium and osteogenic differentiation medium without EVs were used as controls. Microarrays will be used for genome-wide transcriptome analysis of differences in pro-osteogenic potential of monocyte-derived EVs, EVs from inactive osteoclast and EVs from resorbing osteoclast.
Results: Stainings of osteoclast-marker TRAcP confirmed the formation of osteoclasts. Osteoclasts on hydroxyapatite resorbed the coating. Electron microscopy and nanoparticle tracking analysis showed EVs between 50 and 400 nm isolated from the conditioned medium. Additionally western blotting validated the presence of EVs.Our preliminary data show that osteoclast-derived EVs upregulated the expression of osteogenic marker gene RunX2 in AT-MSCs. The microarray data is currently being processed.
Conclusion: We have successfully isolated EVs from monocytes, inactive and resorbing osteoclasts. Our preliminary transcriptomics data show that osteoclast-derived EVs have a pro-osteogenic effect in AT-MSCs.
Authors Gebraad and Mannerström contributed equally.

PF06.03

Characterisation of extracellular vesicle production during leukaemic differentiation

Heather M. Duncan[1], Isabelle Laverdière[2], Héloïse Frison[3] and Kolja Eppert[4]

[1]Division of Experimental Medicine, McGill University, Montreal, Canada; [2]Faculty of Pharmacy, Laval University, Quebec, Canada; [3]BioLegend; [4]Deptartment of Paediatrics, McGill University, Montreal, Canada

Introduction: In acute myeloid leukaemia (AML), leukaemic stem cells (LSCs) are resistant to therapy and lead to relapse. While a growing body of evidence demonstrates that extracellular vesicles (EVs) from leukaemic cells promote disease progression and therapy resistance in AML, little is known about the role of EVs produced by the LSCs. We aim to quantify and characterise the content of EVs released by both the LSC-enriched and differentiated cell populations of primary human AML.
Methods: EVs were isolated from cell-conditioned media by ultracentrifugation, quantified by nanoparticle tracking analysis and stained using lipophilic dye PKH67. Uptake by the same cells was quantified by flow cytometry.
Results: Preliminary experiments have been performed using a primary human AML sample that maintains hierarchical organisation, including a functional LSC population, during *in vitro* culture. We have successfully isolated EVs, quantified them, and observed uptake by AML cells, confirming the functional nature of these EVs. Microscopy was also used to confirm uptake of EVs visualised as distinct foci in recipient cells. Proteomic analysis of the cargo of EVs produced by LSC and more differentiated AML cellsis ongoing.
Conclusion: In primary AML samples, microscopy and imaging flow cytometry can be used to determine EV uptake and co-localisation of markers from EV producing cells with $PKH67^{+}$ foci in recipient cells. We are currently purifying EVs from LSC-enriched ($CD34^{+}/CD38^{-}$) and blast ($CD34^{-}$) cell populations isolated by flow cytometry and performing proteomic analysis to contrast the contents of EVs from these two cell populations. Results of this analysis will be presented at the meeting.

PF06.04

PTEN controls exportation of membrane-bounded proteins including DSCAM and Megf10 via regulating exosome secretion pathway

Nobuhiko Tachibana[1], Robert Cantrup[2], Rajiv Dixit[3] and Carol Schuurmans[4]

[1]University of Calgary, Sunnybrook Health Sciences Centre, Calgary, Canada; [2]University of Calgary, Canada; [3]Sunnybrook Health Science Centre, Calgary, Canada; [4]Sunnybrook Research Institute, University of Toronto and Biochemistry and Molecular Biology Deparment, University of Calgary, Canada

In the retina, neurons of the same type are precisely positioned in two orthogonal planes, in the radial plane, like-neurons are located in specific strata, while in the horizontal/tangential plane, they are evenly distributed in non-random arrays known as mosaics. We found that the retina-specific conditional knockout (cKO) of *Pten*, encoding an intracellular phosphatase, perturbs the mosaic patterning of dopaminergic amacrine cells, phenocopying *Dscam* mutants.It is unclear how cell surface adhesion molecules, such as Dscam, or intracellular molecules, such as Pten, operate at a distance to repulse "like" cells so as to maintain cellular mosaics. We found that Dscam is secreted in retinal extracellular vesicles, while others found that mutations in *Dscam* that block its secretion also perturb amacrine cell mosaics. We thus suggest that Dscam may create extracellular repulsive gradients to control amacrine cell somal positioning, and furthermore, suggest that *Pten* may control this secretion. Indeed, we found that the number of Dscam puncta, speculated to be Dscam-packed intracellular vesicles, is elevated in *Pten* cKO dopaminergic amacrine cell, suggesting that *Pten* controls the processing of Dscam protein. Moreover, the amount of truncated Dscam packaged in large extracellular vesicles is reduced in *Pten* mutant retinas. Finally, for the critical functional test of whether EV secretion of proteins is required to establish amacrine cell mosaics, we manipulated nSmase2 (neutral sphingomyelinase 2 encoded by *Smpd3*), a major biogenetic pathway. Strikingly, electroporation of *Smpd3* into retinal progenitors, which increases EV secretion, decreased the number of dopaminergic amacrine cells in the vicinity of the electroporated patch, while knockdown using sh-*Smpd3* caused amacrine cell clumping. Taken together, our data supports the idea that *Pten* controls amacrine cell spacing by controlling EV-mediated secretion of cell adhesion molecules such as Dscam.

PF06.05

Extracellular vesicles modulate BMP signalling during early embryogenesis

Thomas Draebing, Jana Heigwer, Lonny Juergensen, Hugo A. Katus and David Hassel

Department of Internal Medicine III, University Hospital Heidelberg, Heidelberg, Germany

Introduction: Bone morphogenetic proteins (BMPs) are essential paracrine regulators of the formation of nearly every organ. The response to BMP signalling in target cells is determined by the BMP concentration in the surrounding extracellular space. It has been established for over 50 years that BMP forms gradients to achieve tissue patterning. But so far little is known of how these gradients form. Recent theoretical models and first experimental observations hinted at a role of vesicles in morphogen gradient formation.
Methods: We used zebrafish embryos as an *in vivo* source for EVs secreted during development. EVs were purified using an ultracentrifugation-based method. BMP2/4 presence in EVs was verified by western blotting. The ability of EVs to activate BMP-dependent transcription was measured by a dual luciferase activity assay. EV-secretion was inhibited by morpholino-based knockdown of Rab11 and Rab35 and quantified by nanoparticle tracking analysis. *In vivo* BMP signalling activity was analysed with *in situ* hybridisation and qPCR of nkx2.5.
Results: We were able to observe the presence of BMP2/4 in EVs purified from zebrafish embryos at the end of gastrulation, when BMP2/4 induces the cardiac mesoderm. By analysing EVs from the endodermic cell line End2, we could show that at least part of the EV-delivered BMP2/4 originates from the endoderm, which is known as the source of BMP2 during late gastrulation and early somite stages. Strikingly, EVs of both origins were able to significantly activate BMP-dependent transcriptional responses. Knockdown of Rab11 and Rab35 in zebrafish embryos reduced the amount of secreted EVs significantly. The expression of the BMP target gene nkx2.5, which is a cardiac lineage marker, was strongly reduced upon Rab11/Rab35 knockdown coinciding with a higher fraction of embryos showing a dorsalisation phenotype, both signs for dysregulated BMP signalling.
Conclusion: Delivery of BMP in EVs is essential to ensure correct embryonic development, indicating a role of EVs in morphogen gradient formation.

PF06.06

Glycan profiling analysis of extracellular vesicles from mesenchymal stem cells (MSCs) and osteogenic MSCs

Asako Shimoda and Kazunari Akiyoshi

Graduate School of Engineering, Kyoto University, Kyoto, Japan

Introduction: Extracellular vesicles (EVs) are released from various cells and play an important role in cellular communication. Various molecules including proteins, lipids, DNA, and micro RNA are contained in EVs, and transfer them between cells. Recent studies reported that the analysis of glycan profiles of EVs provide their biophysical functions such as cellular recognition, protein sorting, and so on. Here, we analysed glycan profiles of EVs from different types of human cell lines (MSCs and osteogenic MSCs) using lectin arrays and compared their differences.
Methods: EVs were isolated from adipose-derived stem cells (ADSCs). To induce osteogenic differentiation, ADSCs were cultured in osteogenic media for 21 days. In addition, EV-like vesicles known as matrix vesicles (MVs), released by osteoblasts to induce mineralisation, were isolated from the extracellular matrix (ECM) after 21 days of differentiation. The EVs from both cells or MVs were characterised by immunoblotting, cytokine arrays, transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and lectin microarray analysis.
Results: We obtained 150–200 nm-sized EVs from both ADSCs and osteogenic ADSCs. Exosomal marker (CD81) was detected, and several cytokines that are related with osteogenic differentiation were found in osteogenic ADSCs-derived EVs. While the size and morphology of MVs from ECM were similar to these of EVs, alkaline phosphatase (ALP) activity, a marker for osteogenic activity was significantly higher in MVs. In glycan profiling analysis, we found that α-2,6 sialic acids were highly enriched in EVs compared with original cell membranes, and the cellular uptake of EVs was influenced by the surface sialic acids moiety of the EVs. Moreover, osteogenic MSC-EVs and MVs showed different glycan profiles, indicating that glycan profiles reflect the biogenesis and cell differentiation.
Conclusion: In this study, we revealed that the analysis of glycan profiles of EVs using lectin microarray provides useful information including cell interaction, differentiation, and biogenesis.

PF06.07

Mechanical force accelerates lung development via release of extracellular vesicles

Tanbir Najrana[1], Laura Goldberg[2], Peter J. Quesenberry[2] and Juan Sanchez-Esteban[1]

[1]Department of Pediatrics, Division of Neonatology, Women & Infants Hospital of Rhode Island, RI, USA; [2]Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital, RI, USA

Introduction: Lung underdevelopment secondary to extreme prematurity and pulmonary hypoplasia can cause significant morbidity and mortality to the neonatal population. Currently, there are no effective interventions to accelerate lung development. Mechanical forces generated inside the foetal lung are critical for normal lung development. However, the mechanisms by which mechanical signals stimulate lung development are not fully characterised. Extracellular vesicles (EVs), including exosomes and microvesicles, are small, membrane-bound particles, increasingly recognised as a novel mode of cell-to-cell communication. However, the role of EVs in

foetal lung development is unexplored. Hypothesis: Mechanical signals promote foetal lung development via release of EVs.
Methods: EVs were isolated from E18.5 mouse foetal lungs using differential centrifugation steps. Size and concentration of EVs were measured by nanoparticle tracking analysis. Purity of EVs was analysed by western blot and flow cytometry using anti-CD63 and anti-CD9 antibodies. Isolated E18.5 mouse epithelial cells were cultured on Bioflex plates coated with laminin and exposed to 5% cyclic stretch (to mimic mechanical forces in lung development) for 24 h using the Flexercell Strain Unit.
Results: More than 80% of the EVs isolated from the foetal lung fluids have a diameter of around 100 nm and tetraspanins surface markers including CD9 and CD63, consistent with exosomes. Mechanical stretch of foetal epithelial cells increased release of EVs by 2.4 fold when compared to controls. Moreover, incubation of primary foetal epithelial cells with EVs released from stretched cells or from EVs isolated from foetal lungs promoted type I epithelial cell differentiation.
Conclusion: EVs are present in the lumen of the foetal lung. Mechanical signals release EVs that are important for differentiation of foetal type I epithelial cells. Future studies will test this hypothesis using ex vivo and *in vivo* models

PF06.08

Pancreatic cancer ExoNet

Carolina de Freitas Ruivo[1], Tiago Gama[1], Carlos Melo[2], José Machado[3] and Sónia Melo[3]

[1]i3S – Instituto de Investigação e Inovação em Saúde; [2]The Gurdon Institute, University of Cambridge, United Kingdom; [3]i3S – Ipatimup

Please see OPT01.04

PF06.09

Contribution of extracellular vesicles from adult-derived human liver stem cells to the correction of urea cycle disorders

Catherine Lombard[1], Jiun-Pang Huang[2], Joachim Ravau[1] and Etienne Sokal[3]

[1]Université Catholique de Louvain, IREC-PEDI; [2]Chang Gung University, Taoyuan City, Taiwan; [3]St Luc Hospital and Université Catholique de Louvain, IREC-PEDI

Introduction: Adult-derived human liver stem cells (ADHLSCs) are currently in clinical development for the treatment of urea cycle disorders (UCD). Clinical and preclinical data seem to indicate a higher clinical effect than what could be expected from the number of cells that have engrafted, suggesting that other mechanisms may be at play. We have previously demonstrated that ADHLSCs produce extracellular vesicles (EVs, i.e. microparticles (MPs) and exosomes (EXO)), which have been shown to mediate intracellular communication in other systems by delivering proteins, lipids and/or genetic information (coding and non-coding RNAs) to recipient cells. Therefore, the aim of this study was to determine the precise role of EVs in ADHLSC-mediated correction of UCD.
Methods: ADHLSCs were cultured for 2 days in DMEM supplemented with 10% EXO-free FBS and 1% P/S. The conditioned medium was collected, and MP and EXO fractions were harvested by serial ultracentrifugation. Transmission electron microscopy (TEM), western blotting and nanoparticle tracking analysis were used to evaluate the presence, purity and abundance of MP and EXO. RNA from EVs was stained with SytoRNA, which only fluoresces upon integration into RNA, to investigate RNA transfer from EVs to rat hepatocytes. Droplet digital PCR (ddPCR) was performed on RNA extracted from the MP and EXO as well as rat hepatocytes previously incubated with EVs to investigate the presence of human mRNAs of interest.
Results: We confirmed that ADHLSCs produce both MP and EXO. Characterisation of the mRNA by ddPCR showed expression of ASL, ASS and CPS1 in EVs, mainly in MPs. SytoRNA staining of the EV RNA allowed us to show transfer of EV RNA to over 60% of rat hepatocytes *in vitro*. Finally, we demonstrated transfer of human mRNAs of interest from EVs to rat hepatocytes using ddPCR
Conclusion: In summary, our study shows that ADHLSC-derived EVs contain mRNA encoding for some of the deficient enzymes in UCD and are capable of transfering their mRNA content to recipient cells. mRNA transfer via EVs may therefore be one of the modes of action of ADHLSCs in UCD.

PF06.10

Osteoblast-secreted extracellular vesicles stimulate the expansion of CD34+ human umbilical cord blood cells

Jess Morhayim, Jeroen van de Peppel, Eric Braakman, Elwin Rombouts, Mariette ter Borg, Bram van der Eerden, Andre van Wijnen, Jan Cornelissen and Johannes P. van Leeuwen

Erasmus MC

Introduction: Osteolineage cells represent one of the critical bone marrow niche components that regulate self-renewal and differentiation of hematopoietic stem and progenitor cells (HSPCs). Recent studies demonstrated that extracellular vesicles (EVs) regulate stem cell development via horizontal transfer of bioactive cargo. In the present study, we focused on the characterisation of human osteoblast-derived EV-miRNAs and investigated their implications on HSPC-osteolineage-cell crosstalk.
Methods: We used human pre-osteoblasts (SV-HFO cells) to isolate EVs by a series of ultracentrifugation steps. We elucidated the overrepresented EV-miRNAs by comparing parental cell- and EV-miRNA profiles using next-generation sequencing. We performed *in silico* target prediction analyses to delineate candidate hematopoietic development pathways affected by osteoblast-EVs and subsequently verified our results with *in vitro* biochemical analyses. We investigated the potency of osteoblast-EVs to promote *ex vivo* expansion of human umbilical cord blood (UCB)-derived $CD34^+$ HSPCs and subsets by enumeration using single-platform flow cytometry. We further verified the functionality of the expanded cells *in vivo* by performing xenogeneic transplantation in immunodeficient mice.
Results: Using next-generation sequencing we show that osteoblast-EVs contain highly abundant miRNAs specifically enriched in EVs, including critical regulators of hematopoietic proliferation (e.g. miR-29a). EV treatment of human umbilical cord blood-derived $CD34^+$ HSPCs alters the expression of candidate miRNA targets, such as *HBP1, BCL2* and *PTEN*. Furthermore, EVs enhance proliferation of $CD34^+$ cells (2-fold, $p < 0.01$) and their immature subsets (>2-fold, $p < 0.005$) in growth factor-driven *ex vivo* expansion cultures. Importantly, EV-expanded cells retain their differentiation capacity *in vitro* and successfully engraft *in vivo*.
Conclusion: In this study, we demonstrate a novel osteoblast-derived EV-mediated mechanism for regulation of HSPC proliferation and expansion. These discoveries provide a foundation for the utilisation of EV-miRNAs for the development of UCB-HSPC expansion strategies to treat haematological disorders.

PF06.11

Withdrawn at author's request.

PF06.12

A rapid microflow analysis of cancer stem cell surface proteins in circulating exosomes from breast cancer patients

Golam Kibria[1], Erika Ramos[2], Clifford Harding[1], Jan Lötvall[3] and Huiping Liu[2]

[1]Case Western Reserve University, OH, USA; [2]Northwestern University, CA, USA; [3]Krefting Research Centre, University of Gothenburg, Sweden

Circulating exosomes provide a promising approach to assess novel and dynamic biomarkers in human disease, due to their stability, accessibility and representation of molecules from source cells. However, this potential has been stymied by lack of approaches for molecular profiling of individual exosomes, which have a diameter of 30–150 nm. Existing approaches to exosome characterisation include electron microscopy, nanoparticle tracking analysis, protein and RNA analyses for collective exosomes (immunoblotting, mass spec, RNA array, PCR and sequencing etc.), and other biochemical assays. However, most of these approaches are often not feasible to rapidly assess the heterogenous profiles of individual exosomes. Here we report a rapid microflow analysis approach for high throughput profiling of surface proteins at a single exosome level, a major challenge to moving the field of exosome-based biomarkers forward (1).

Cancer stem cells (CSCs) are a subpopulation of cancer cells with stem cell-like properties of self-renewal and tumorigenesis. CSCs, often considered the root of cancer, seeds of metastasis, and sources of therapy resistance, might communicate with the microenvironment through secreted circulating exosomes. We hypothesised that circulating exosomes harbour surface protein markers of CSCs and correlate with the status of these cells *in vivo* and the predictive outcome of cancer patients. Using a micro flow cytometer Apogee, we optimised the microflow analyses of CSC markers CD44 and CD47, of circulating exosomes isolated from the blood of both breast cancer patients and healthy populations. Our studies show a differential CD47 expression in blood-purified individual circulating exosomes that is associated with breast cancer status, demonstrating a great potential of individual exosome profiles in biomarker discovery. The sensitive and high throughput platform of single exosome analysis can also be applied to characterising exosomes derived from other patient fluids.

Reference

1. Kibria G et al., Sci Rep. 2016; 6: 36502.

PF06.13

Benefits of human neural stem cell derived extracellular vesicles surpass those of mesenchymal stem cell derived vesicles in a murine embolic stroke model

Robin Webb[1], Shelley Scoville[1], Tyler Thompson[1], Nasrul Hoda[2] and Steven Stice[1]

[1]ArunA Biomedical; [2]Augusta University, GA, USA

Introduction: Mesenchymal stem cell (MSC)-derived extracellular vesicles (EV) have provided benefit in stroke animal models. However, exosome cargos are cell type specific and the parental cell line plays a prodigious role in the biological properties of the resultant vesicles. Thus, compared to MSCs, neural sourced EVs may provide additional benefit to the injured brain. To test this hypothesis, EVs were derived from MSCs and neural progenitor cells (NPCs), originating from the same parent human pluripotent stem cell line, enabling us to determine the *in vivo* response in a stroke model to EVs from different cell types that have the same genetic background.

Methods: Defined serum-free media conditioned by MSC or NSC were thawed, subjected to dead end filtration, and enriched by ultrafiltration. EVs were quantified using NanoSight. 2.7×10^{11} NPC and MSC vesicles/kg (±10%) doses, referred to as NPEX and MSCEX, respectively, were stored in individual dose aliquots at −20°C. After thaw, these were administered by tail vein injection (N = 12 mice/group) at 2, 14 and 28 h post embolic stroke. Control animals received PBS injections at all timepoints, blood collection for flow cytometry followed by euthanasia occurred 96 h post-stroke.

Results and Conclusion: Preliminary proteomics data indicated overlapping but divergent protein profiles between MSCEX and NPEX in angiogenic and neuroprotective proteins. Enrichment of these proteins in NPEX led us to hypothesise that these EVs may provide enhanced benefits *in vivo*. In the mouse embolic stroke model, NPEX decreased mortality by 17%. Sensorimotor function (adhesive tape test), and neurological deficit score were improved by NPEX treatment, with animals that received MSCEX performing like controls. Infarct volume (% control) was significantly decreased following NPEX treatment, but unchanged by MSCEX. NPEX increased circulating regulatory T-cells (relative to both MSCEX and control treated groups), as well as anti-inflammatory M2 macrophages, while decreased inflammatory T-cells were detected. Taken together, these data indicate that NPEX provided molecular and behavioural benefits that surpassed those provided by MSCEX following stroke, supporting a role for NPC derived EVs as a biological therapeutic following stroke in humans.

PF06.14

Extracellular vesicles derived from human umbilical cord perivascular cells (HUCPVCs): a potential non-cell source for regenerative therapy

Nechama Lipton[1], Paula Mackie[1], Kathryn Lye[1], Lianet Lopez[1], Farwah Iqbal[1,2], Peter Szaraz[1], Shlomit Kenigsberg[1], Denis Gallagher[1], Andree S. Gauthier-Fisher[1] and Clifford Librach[1,3,4]

[1]CReATE Fertility Centre; [2]Department of Physiology, University of Toronto, Toronto, Canada; [3]Department of Obstetrics and Gynaecology, Department of Physiology, Institute of Medical Sciences, University of Toronto, Toronto, Canada; [4]Department of Gynecology, Women's College Hospital

Introduction: Intercellular transfer of extracellular vesicles (EVs) may mediatekey paracrine regenerative activities of mesenchymal stem cells (MSCs). First trimester (FTM) and term HUCPVCs are novel sources of MSCs with high regenerative potential. The objective of this study was to optimise methods for efficient production and purification of HUCPVC-EVs and to investigate their potential regenerative properties *in vitro*.

Methods: FTM and term HUCPVCs, as well as human fibroblasts, were expanded in multi-layer flasks using aMEM media containing EV-depleted FBS for 72hrs. EVs were isolated from concentrated conditioned media (CM) using ultracentrifugation (UC), sucrose cushion UC or the ExoEasy kit (Qiagen). The presence, size and morphology of the HUCPVC-derived EVs were determined by transmission electron microscopy (TEM) and Nanoparticle Tracking Analysis (NTA). To visualise EV uptake by target cells, rat endothelial progenitor cells (EPCs) were treated with HUCPVC-derived EVs prelabelled with PKH26 dye. The paracrine angiogenic and neuroprotective properties of HUCPVCs and HUCPVC-EVs were evaluated using the rat aortic ring assay and a murine *in vitro* diabetic neuropathy model.

Results: EVs were successfully isolated from FTM, term HUCPVC and fibroblasts CM but not basal media. UC with a sucrose cushion and ExoEasy kit reduced contaminating proteins in concentrated media compared to UC alone. From TEM, isolated EVs were 30–200 nm with a cup-shaped morphology. Uptake of PKH26-labelled EVs derived from both HUCPVCs and fibroblasts was observed in EPCs. Aortic rings treated with HUCPVCs showed increased mean radial network growth and mean number of loops when compared to untreated networks. Neuronal cultures treated with EVs showed decreased axonal degeneration following exposure to hyperglycemia, and increased neurite outgrowth.

Conclusion: HUCPVCs secrete EVs which can be taken up by EPCs *in vitro*. UC with sucrose cushion or ExoEasy kit isolate purer EV fractions when compared to UC. HUCPVCs display paracrine angiogenic and neuroprotective properties. Experiments utilising purified HUCPVC-derived EVs are ongoing to determine the relative contribution of EVs to these regenerative properties.

Poster Session F07 – EVs in the Central Nervous System
Chairs: TBD and Paula Saa 5:15–6:30 p.m.

PF07.01

Stroke extracellular vesicles express inflammatory markers and induce macrophage activation

Yvonne Couch[1], Naveed Akbar[1], Simon Davis[1], Roman Fischer[1], Kim Wals[1], Alex Dickens[2], Ain Neuhaus[1], Annette Burgess[1], Peter Rothwell[1] and Alastair Buchan[1]

[1]University of Oxford, Oxford, United Kingdom; [2]University of Turku, Helsinki, Finland

Please see OPT02.04

PF07.02

Activated monocyte-derived exosomes stimulate adhesion molecules and cytokines in human brain endothelial cells: role of exosomes in monocyte brain migration

Lynn Pulliam[1], Pranjali Dalvi[2], Norina Tang[2], Peilin Li[1] and Bing Sun[2]

[1]University of California, San Francisco, CA, USA; [2]Veterans Affairs Medical Center

Introduction: Widespread use of antiretroviral therapy (ART) by HIV- infected subjects has improved their health and extended their lives, however, with increased longevity, co-morbidities have become significant. A subset of HIV-infected individuals continues to have chronic immune activation with cognitive impairment in spite of effective therapy.We reported that individuals with HIV infection have a type 1 interferon (IFN) phenotype with elevated circulating lipopolysaccharide (LPS). We modelled this *in vitro* to determine whether monocyte-derived exosomes (exos) would increase adhesion molecules and cytokine expression in human brain microvascular endothelial cells (HBMECs) and thereby facilitate monocyte migration into the brain.
Methods: Monocyte exos were labelled with DiI-C16 and incubated with HBMECs to confirm entry. Monocytes were activated with IFN, LPS or IFN followed by LPS (I/L). Exos were harvested 24 h. later, lysed to isolate total RNA and probed by RT-PCR for adhesion molecules and cytokines. Conditioned media or cells from the activated monocyte exo-treated HBMECs were probed for protein expression. Monocytes were incubated on top of a transwell chamber with HBMECs in the bottom, with or without GW4869, an inhibitor of exo release to determine the impact of exos on monocyte migration. HBMEC mRNA was quantified for adhesion molecules and cytokines by qRT-PCR.
Results: Exos from LPS or I/L-treated monocytes stimulated CCL2, ICAM-1, VCAM, IL-1β and IL-6 gene expression and protein in HBMECs. Monocyte-derived exos were internalised and those stimulated with LPS or I/L, activated NF_kB nuclear translocation. An increase in the migration of LPS or I/L-stimulated monocytes towards HBMECs was observed. Inhibition of exo release significantly normalised the monocyte migration to the level of unstimulated control cells. This was supported by the simultaneous increase in CCL2, ICAM-1, VCAM, IL-1β and IL-6 in HBMECs in the lower chamber of I/L-activated monocytes, the inhibition of exo release notable reduced these activation markers.
Conclusions: In HIV positive individuals with elevated circulating LPS and an IFN profile, exos may play a crucial role in causing brain injury by stimulating chemotaxis of monocytes to brain endothelium.

PF07.03

Withdrawn at author's request.

PF07.04

CNS-derived extracellular vesicles are heterogeneous and adaptive to age and tissue of origin

Sarah M. Fernando[1], Chih Chieh Shu[1], Darren D. Christie[1], Leslie I. Grad[2], Neil R. Cashman[1] and Judith M. Silverman[1]

[1]University of British Columbia, Centre for Brain Health, British Columbia, Canada; [2]Michael Smith Foundation for Health Research, British Columbia, Canada

Introduction: Extracellular vesicles (EVs) are secreted by myriad cells in culture and unicellular organisms, and their identification in mammalian biofluids suggests that vesicle release is occurring at the organism level as well. However, despite clear importance to the understanding of EVs in organismal biology, EVs in solid tissues have received little attention.
Methods: We applied a protocol for primary neuronal cell culture and modified it for the collection of EVs from neural tissues. Exosome (EX) and microvesicle (MV) populations were isolated from frozen whole neural tissues from WT and an ALS mouse model, $SOD1^{G93A}$, by serial centrifugation and purification on a sucrose cushion. Vesicles were phenotyped by flow cytometry on a Miltenyi MACS-Quant using conjugated primary antibodies.
Results: Flow cytometric phenotyping found that the majority of brain and spinal cord EVs are positive for the exosomal marker CD81 and the astrocyte marker GLAST (60% MV and 25% EX), while markers for neurons (NCAM/CD56) were less common (40% MV and 10% EX). CD11b, a microglial marker, was in low abundance (G93A CNS-derived EVs, and this was mostly unchanged by the age and disease status of the mice, in contrast to the significant loading of misfolded SOD1 into $SOD1^{G93A}$ CNS-EVs. Spinal cord vesicles were significantly reduced in GLAST and NCAM/CD56 expression compared to BDEVs, while CD81 and CD11b expression levels were equal between brain and spinal cord vesicles.
Conclusion: These results suggest that microglia contribute little to the brain extracellular vesicle population in young to middle aged mice, while the majority of vesicles are derived from astrocytes. The same is not true for the spinal cord, where a lower percentage of astrocyte marker bearing vesicles contribute to the population. Current work is focused on determining the cell type primarily responsible for releasing misfolded $SOD1^{G93A}$ in EVs in the brain and spinal cord.

PF07.05

Nogo-A as an extracellular vesicle-associated ligand in the central nervous system

Mea M. Holm[1,2], Matteo Egger[1], Danielle van Rossum[1,2], Oliver Weinmann[1,2], Michael Maurer[2], Benjamin Ineichen[1,2], Inge Hermann[3] and Martin E. Schwab[1,2]

[1]ETH Zurich, Zurich, Switzerland; [2]University of Zurich, Zurich, Switzerland; [3]EMPA Swiss Federal Laboratories for Materials Science and Technology, Switzerland

Introduction: Nogo-A is a membrane protein initially identified as a myelin-associated inhibitor of axonal growth and regeneration in the central nervous system (CNS). It has since been discovered that Nogo-A is expressed not only by oligodendrocytes, but also by neurons, in which it plays complex roles in regulating migration, branching and synaptic plasticity. The current view of Nogo-A signalling is that plasma membrane-bound Nogo-A binds to the receptors S1PR2 and/or NgR1 in a multi-subunit complex, thereby requiring a direct cell-to-cell contact. However, the presence of Nogo-A sequences in culture media and cerebrospinal fluid (CSF) has been anecdotally reported and recently found in proteomic studies, raising the possibility of an alternative

signalling mechanism independent of cell-to-cell contact. We therefore sought to investigate if CNS-derived cells secrete Nogo-A in association with extracellular vesicles (EVs) or free in solution, and whether Nogo-A can act as an EV–associated or a soluble ligand in bodily fluids.
Methods: EVs were collected either through ultracentrifugation or through the density gradient method, and analysed through western blotting, nanoparticle tracking and transmission electron microscopy (TEM). For assays of Nogo-A functionality, the fibroblast spreading assay (1) was adapted for use with EV-associated Nogo-A in solution.
Results: We found that Nogo-A is secreted into the supernatant of both neuron- and oligodendrocyte-derived cell cultures, as well as into the CSF of adult rats. TEM analysis with immunogold labelling indicated that Nogo-A is associated specifically with the EV membrane, rather than free in solution or inside the EVs. Furthermore, we found that Nogo-A positive EVs inhibited the spreading of fibroblasts, while Nogo-A negative control EVs did not. The spreading inhibition could be rescued by the addition of a blocker for the Nogo-A receptor S1PR2.
Conclusion: These data show that Nogo-A positive EVs are secreted by CNS cells and can be isolated from the CSF. The EV-associated Nogo-A is functional as a ligand in *in vitro* assays, raising the intriguing possibility of an *in vivo* signalling function, which would have major implications for the administration of anti-Nogo-A antibodies as therapies.

Reference

1. Oertle T et al., *J Neurosci.* 2003; 23: 5393–5406.

PF07.06

Role of exosomes in axon outgrowth

Samar Ahmad[1] and Liliana Attisano[2]

[1]University of Toronto, Toronto, Canada; [2]Department of Biochemistry, University of Toronto, Toronto, Canada

In the central nervous system (CNS), exosomes are involved in inter-neuronal communication and modulate axon outgrowth and axon guidance that are key processes during brain development and injury. Exosomes derived from different sources have different compositions, and thus the origin of exosomes may induce distinct cell responses. Previously, mouse fibroblast (L-cell)-derived (FD) exosomes have been shown to promote cell protrusion and motility in human breast cancer cell lines. Thus, to begin to assess the differential activity of exosomes of distinct origins in the CNS, the function of FD exosomes in axon outgrowth was investigated in this preliminary study. Treatment of isolated primary mouse embryonic cortical neurons with exosomes purified by ultracentrifugation, promoted axon outgrowth. Moreover, the effect of exosomes isolated through various purification methods on axon outgrowth was evaluated. Though further investigation is required to examine the underlying mechanism of the exosome-induced axon outgrowth, the current preliminary study highlights the potential role of FD exosomes on axon outgrowth. Altogether, this will increase our understanding of exosome activity on neurons and the potential of exosomes to overcome developmental defects and injury in the CNS.

PF07.07

Exosomal miRNA-induced lincRNA regulates microglial phagocytosis: implications for morphine-mediated potentiation of neurodegeneration

Guoku Hu, Ke Liao, Fang Niu and Shilpa Buch

Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, NE, USA

Introduction: Opioids such as morphine are the most potent and efficacious drugs currently available for pain management. Both *in vitro* and *in vivo* studies have demonstrated that morphine potentiates the neurodegenerative effects of HIV in the central nervous system (CNS). Impairment of microglial functions such as phagocytosis and activation has been implicated as in mediating neurodegeneration underlying various CNS diseases. In recent times, roles of long intergenic noncoding RNAs (lincRNAs) in regulating cellular processes is gaining attention. While lincRNAs are known to maintain cellular homeostasis, dysregulation of their expression by EV has been implicated in regulation of a wide array of genes including those controlling phagocytosis. Based on this we hypothesised that EVs released from morphine exposed astrocytes can be taken up by microglial cells leading in turn, to impaired microglial phagocytosis via the TLR-NF-kB axis-induced lincRNA-Cox2.
Methods: Mouse primary astrocytes and human A172 astrocytoma cells were exposed to morphine (10 μM) followed by isolation of EVs using the standard differential ultracentrifugation technique. Transmission electron microscopy, NanoSight and western blot analyses were used to characterise EVs. Expression of lincRNA-Cox2 in EV-treated BV2 cells and mouse primary microglial cells was examined by qPCR. Microglial phagocytosis was assessed by uptake of fluorescently labelled latex beads. *In vivo* studies involved intranasal delivery of lincRNA-Cox2 siRNA to mice that were administered morphine.
Results: EVs released from morphine exposed astrocytes demonstrated upregulation of miR-138, which in turn, was shown to bind to the endosomal TLR7 in microglia, leading to activation of NF-kB pathway. This in turn, resulted in upregulation of lincRNA-Cox2, leading to impaired microglial phagocytosis. Intranasal delivery of lincRNA-Cox2 siRNA ameliorated microglial phagocytic activity in morphine-treated mice.
Conclusion: Exposure of microglial cells to EVs released from morphine-exposed astrocytes resulted in impaired phagocytic function via the TLR7-NF-kB-lincRNA-Cox2 axis. These findings have ramifications for the development of EV-loaded RNA drug target(s) as therapeutics for neurodegenerative disorders associated with opiate abuse.

PF07.08

Exosomes derived from ACE2-overexpressing endothelial progenitor cells protect neurons from hemolysate-induced apoptosis and inflammation

Jinju Wang[1], Qunwen Pan[2], Yanfang Chen[1], Bin Zhao[2], Xiaotang Ma[2] and Ji Bihl[1]

[1]Wright State University, OH, USA; [2]Affiliated Hospital of Guangdong Medical University, Guangdong, China

Introduction: We have previously demonstrated that angiotensin converting enzyme 2 (ACE2)/angiotensin (Ang)-(1-7)/Mas pathway has therapeutic effects on intracerebral haemorrhagic stroke through inhibiting necrosis factor nuclear factor-KappaB (NFκB) inflammatory pathway. More recently, we found that exosomes of endothelial progenitor cells (EPCs-EXs) could protect neurons from hypoxia/reoxygenation-induced apoptosis. In this study, we tested whether EXs from ACE2 primed EPCs (ACE2-EPC-EXs) have combined beneficial effects on neurons in an *in vitro* haemorrhagic model induced by hemolysate.
Methods: EPCs cultured from the bone marrow of C57BL/6 mice were transfected with Lenti-ACE2 (at 5×10^6 infection-forming units). EXs were collected from the culture medium of EPCs by ultracentrifuge. Neuron 2a cells were pretreated with vehicle (PBS), EPC-EXs or ACE2-EPC-EXs (50 μg/ml) for 12 h, and then incubated with hemolysate (10%) for 6 h. Hemolysate was prepared from the fresh mouse arterial blood. The apoptosis of neurons was determined by flow cytometry. The expressions of NFκB, inhibitor of κBα (IκBα), cyclooxygenase-2 (COX-2) and interleukin-1β (IL-1β) were confirmed by Western blot.
Results: Hemolysate induced neuronal apoptosis (by 40%), which was accompanied by up-regulations of NFκB (~4-fold), COX-2 (by 44%) and IL-1β (~2.8-fold), but a down-regulation of IκBα (by 50%). Pretreatment with ACE2-EPC-EXswas more effective on decreasing hemolysate-induced neuronal apoptosis (by 25 ± 2.8% and 34 ± 4.2%, ACE2-EPC-EXs *vs.* EPC-EXs, $p < 0.05$). Similarly, the hemolysate-induced effects on NFκB, COX-2 and IL-1β, and IκBα expression were more inhibited by ACE2-EPC-EXs (by 25–48%, ACE2-EPC-EXs *vs.* EPC-EXs, $p < 0.05$).
Conclusion: Data suggest that ACE2-EPC-EXs have better efficacy than EPC-EXs in protecting neurons from hemolysate-induced apoptosis and inflammation.

PF07.09

Proteomic analysis of microvesicles from CSF of multiple sclerosis patients

Antonella D'Ambrosio[1], Sandra Columba Cabezas[1], Serena Camerini[1], Maria Luisa Casella[1], Marco Crescenzi[1], Marco Puthenparampil[2], Silvia Zamboni[1], Marco Diociaiuti[1], Francesca Aloisi[1], Paolo Gallo[3] and Paola Margutti[1]

[1]Istituto Superiore di Sanità; [2]Department of Neuroscience DNS, University of Padua, Padua, Italy; [3]Multiple Sclerosis Centre, Department of Neurosciences DNS, University Hospital – Medical School

Introduction: Multiple sclerosis (MS) is an inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS). Emerging evidence indicates that different types of CNS cells release high numbers of microvesicles (MVs) in the cerebrospinal fluid (CSF). MVs, sharing the same antigenic repertoire as their parental cells, may dynamically reflect pathologic mechanisms of CNS damage representing a novel class of circulating biomarkers. The main goal of this study is to identify CNS biomarkers related to brain damage in relapsing-remitting MS and in clinically isolated syndrome (CIS), characterised by a single neurological episode suggestive of MS and a high probability to convert to clinically definite MS.
Methods: We performed a proteomics-based biomarker discovery study in the CSF of two CIS patients, four relapsing–remitting MS (RRMS) patients and two healthy subjects.The diagnostic work-up included MRI, visual evoked potentials and CSF examination. CSF-derived MVs were purified by size using Sephacryl S-500 gel filtration column and concentrated by ultracentrifugation. Proteomic analyses of purified CNS-derived MVs were carried out through pre-fractionation of MV protein samples by one dimensional SDS-PAGE followed by LC-MS/MS. Peptide identification was performed using the NCBI database.
Summary: For the first time, total CSF as well as purified CSF-derived MVs from CIS and RRMS patients have been analysed by a "proteomic phenotyping" approach. In the preliminary analyses, two proteins were detected exclusively in one of the two CIS patients with BBB damage but not in RRMS patients: neuronal cell adhesion molecule (NCAM-140), derived from purified MVs, is related to remyelination and Beta-Ala-His dipeptidase, derived from total CSF, was previously identified as a predictive biomarker of CIS to MS conversion.
Conclusion: Further studies in a larger patient cohort will be performed to validate the potential relevance of these two proteins as biomarkers associated to brain damage in early MS phases.

PF07.10

Primary culture photoreceptors release functional extracellular vesicles

Aikaterini Kalargyrou[1], Benjamin Davis[1], Enrico Cristante[1], Emma West[1], Anai Anai Gonzalez-Cordero[2], Anastasios Georgiadis[1], Matt Hayes[3], Francesca Cordeiro[4], Sander Smith[1], Robin Ali[1] and Rachael Pearson[1]

[1]UCL Institute of Ophthalmology; [2]Institute of Ophthalmology; [3]UCL Institute of Ophthalmology – EM Unit/Imaging SRF; [4]Institute of Ophthalmology – Visual Neuroscience

Introduction: Extracellular vesicles (EVs) are key players of intercellular communication, enabling the transfer of proteins, lipids and RNA between cells. The nervous system requires tightly regulated exchanges between sensory and motor neurons, interneurons and glial cells. Recent studies have attributed some of these exchanges to EVs and they have been found to modulate diverse processes, including neuronal survival and degeneration, the microglial immune response, synapse assembly and plasticity. Very little is known about the presence and potential roles of EVs in the neuroretina, in either health or disease. As a first step, we investigated whether photoreceptors (PRs) in the normal mammalian retina have the capacity to make and release EVs.
Methods: CD73+ primary PRs were isolated from postnatal day P8 wildtype mouse retinae using MACS and cultured for 14 days. EVs were isolated from culture medium using differential UC. Pellets from 10,000*g* and 100,000*g* spins analysed with DLS and TEM. EV composition analysed using western blot, dot-blot and RTqPCR. Functional read-outs utilised a transwell co-culture system with a Cre-loxP recombination read-out.
Results: P8 rod PRs survive in culture conditions without serum and release EVs within 72 h. Protein profiling of 100,000*g* pellets revealed expression of Alix and Tsg101 but not CD63. RTqPCR shows enrichment for rod specific mRNA though at the lower limits of technical detection. DLS revealed distinct populations at diameters of 100 nm, 300–500 nm and 1000 nm, which were further confirmed with TEM. To assess whether PR-derived EVs are functional, we employed a transwell co-culture system with Cre+ PRs placed in the top insert and dissociated Ai9 TdT^{floxed} dissociated retina cultured at the bottom of the well. TdT+ microglia and astrocytes were observed after 14 days of incubation with Cre+ PRs while no recombination was seen in control PRs.
Conclusion: Primary culture PRs release EVs with morphological and molecular profiles typical of neuronal EVs and contain photoreceptor specific RNA and/or protein, which may serve as marker of EV cell origin. Further work is required to determine whether these EVs are being taken up by other cells in the retina. Limitations in PR survival currently preclude any conclusion regarding communication with other PRs.

PF07.11

Extracellular vesicles as mediators of periphery-to-brain communication: relevance for stress-induced neuropsychiatric disorders

Giorgio Bergamini[1], Hannes Sigrist[1], Sandra Auer[1], Tobias Suter[2], Erich Seifritz[3] and Christopher Pryce[1]

[1]Preclinical Laboratory for Translational Research into Affective Disorders, Psychiatric Hospital, University of Zurich, Zurich, Switzerland; [2]Clinical Immunology, University Hospital Zurich, Zurich, Switzerland; [3]Psychiatric Hospital, University of Zurich, Zurich, Switzerland

Introduction: There is evidence that inflammation is important in the aetiology of several psychiatric disorders, including major depressive disorder (MDD). Psychosocial stress, a major risk factor for MDD, is a primary source of peripheral low-grade inflammation. A state of chronic inflammation can induce MDD symptoms through a multiplicity of effects on the functioning of brain neurocircuitry, including the dopaminergic system. Understanding of the aetio-pathophysiological pathways mediating between stress, inflammation, altered brain function and psychopathology is currently limited. Interestingly, extracellular vesicles (EVs) derived from hematopoietic cells can deliver miRNAs to CNS cells during inflammatory conditions. The aim of this study is to (a) investigate the effects of psychosocial stress on the peripheral immune system and on dopamine (DA) neurons, and (b) assess if stress modifies blood EVs miRNA content and their communication to brain DA cells.
Methods: Mice exposed to chronic social defeat (CSD) stress are assessed for depression relevant-behaviours, peripheral inflammation markers and dopamine system de-regulation. To investigate the effect of CSD on EVs, plasma EVs are isolated and miRNA content is analysed using qPCR. To investigate the hypothesis that stress stimulates EVs-mediated periphery-to-brain communication, Vav1-iCre x Rosa26-GFP mice are used. Neurons receiving EVs cargo from (Vav1+) hematopoietic cells are identified by Cre-mediated GFP expression.
Results: Mice exposed to CSD exhibit increased splenic granulocytes, inflammatory monocytes and T helper 17 cells. The immune response co-occurs with attenuation of dopamine signalling and depression-relevant behaviours. Future experiments will examine if (a) CSD affects the miRNA cargo of blood EVs and (b) CSD-induced peripheral inflammation stimulates EVs-mediated transfer of RNA from blood immune cells to the brain's DA neurons, and affects DA cells gene expression.
Conclusion: These proposed experiments would serve to identify EVs-RNA peripheral biomarkers, demonstrate their pathophysiological importance in MDD-relevant brain and behavioural dysfunctions, and allow for the identification of potential therapeutic targets for stress-induced behavioural disorders.

PF07.12

Misfolded proteins are carried by leucocyte-derived microvesicles in amiothrophic lateral sclerosis

Daisy Sproviero[1], Sabrina La Salvia[1], Federico Colombo[2], Marta Giannini[1], Luca Diamanti[3], Paola Bini[3], Orietta Pansarasa[1], Laura Porretti[4] and Cristina Cereda[1]

[1]Genomic and post-Genomic Centre, IRCCS, C. Mondino National Institute of Neurology Foundation; [2]Istituto Clinico Humanitas IRCCS; [3]Neurology Department, IRCCS, C. Mondino National Institute of Neurology Foundation; [4]Flow Cytometry Centre and Experimental Hepatology Service, IRCCS Ca' Granda Ospedale Maggiore Policlinico Foundation

Introduction: The lack of biomarkers in amiothrophic lateral sclerosis (ALS) makes impossible to determine the stage of the illness in patients and that delays therapeutic trials. "Misfolded" proteins (SOD1, TDP-43 and FUS) are templates for the formation of protein oligomers that accumulate and interfere with neuronal function, eventually leading to cell death. Blood contains microvesicles (MVs), vesicles that bud directly from the plasma membrane and "misfolded" proteins have been found in plasma MVs of ALS patients highlighting a connection between motoneurons and peripheral blood. The aim of the present study was to characterise MVs in plasma of ALS patients, in order to discover a new mechanism in disease progression.
Methods: Microvesicles were isolated from plasma of 40 ALS, 28 AD patients and 36 healthy volunteers by ultracentrifugation. Markers for MVs of leucocyte (CD45), endothelial (CD31), platelet (CD61), erythrocyte (CD235a) derivation and Annexin V were used for flow cytometry. CD45 MVs were separated by immunoprecipitation and SOD1, TDP43, FUS protein level was investigated in whole lysate and CD45 MVs by WB.
Results: Higher misfolded SOD-1 was found in plasma derived MVs of ALS patients compared to healthy donors (ANOVA test, $p < 0.0001$), but no difference in TDP43. Among four different markers detected by flow cytometry, LMVs (leucocyte-derived microvesicles-CD45 MVs) were mostly present in ALS patients compared to Alzheimer's disease (AD) patients and healthy donors (ANOVA test, $p < 0.001$). The percentage of LMVs was inversely correlated with the progression rate in fast progressing patients (Spearman r = −0.52, $p = 0.02$) and directly correlated with the progression rate in slow progressing patients (Spearman r = 0.38, $p = 0.038$). Isolated LMVs of slow progressing ALS patients carried more misfolded SOD1 than the ones of healthy donors and fast progressors and misfolded SOD1 protein level was strongly associated with the percentage of LMVs in slow progressing patients (Pearson r = 0.71, $p = 0.0029$).
Conclusion: Leucocyte-derived MVs are regulated by the rate of disease progression in ALS patients and can act as "carriers" of misfolded proteins, main cause of disease propagation.

PF07.13

Proteome analysis of cochlear pericyte-derived exosomes in normoxic and hypoxic condition

Elisa Ghelfi[1], Emil Millet[2], Magda Bortoni[2], Adam Bartos[2], Yohann Grondin[2], Rosalinda Sepulveda[2] and Rick Rogers[2]

[1]Harvard Chan School of Public Health, Department of Environmental Health, MIPS Program, MA, USA; [2]Harvard Chan School of Public Health, MA, USA

Introduction: Ototoxic drugs such as gentamicin induce the formation of free radicals in the inner ear resulting in inflammation and damage to the cochlear cells and microvasculature. Free radicals are also considered the main culprit in noise induced hearing loss. Hypoxia has been shown to occur in loud noise conditions due to blood stagnation and stopped flow, leading to free radicals production and potentiating noise induced hearing loss. The inner ear microvasculature, which is formed by two major vascular beds, the stria vascularis and the spiral ligament (SL) vasculature, exhibits a blood–inner ear barrier, the BLB, which is similar to the blood–brain barrier (BBB). The SL microvasculature and SL pericytes have been shown to share similarity with the brain capillaries. SL pericytes play an important role in maintaining the integrity of the BLB. We investigated if SL pericytes express markers of brain pericytes and if the ototoxic drug gentamicin and hypoxia, induce inflammatory response in SL pericytes. We then investigated the difference in exosome proteomics in normoxia and hypoxia.
Methods: SL pericytes were obtained from Immortomouse®. Cells were first incubated with gentamicin in normoxic and hypoxic conditions and the level of inflammatory response was assayed. FBS exosome depleted conditioned media was used for growing SL pericyte cultures in normoxic and hypoxic conditions. Exosomes were isolated from conditioned medium with basic differential ultracentrifugation and the morphology examined by electron microscope. Exosome proteome was obtained with a LTQ Orbitrap Velos Pro ion-trap mass spectrometer.
Results: SL pericytes showed positive signal for the validated brain pericytes marker CD13. Incubation with the ototoxic drug gentamicin induced SL phagocytic activity and increased the expression of cytokines such as IL-6 and the LIF and MIP-2 and VEGF. The differences in the exosome proteome from normoxic, hypoxic and gentamicin challenged cells was analysed with bioinformatics tools for identifying and visualising enriched GO terms and the proteins function of the exosome proteome.
Summary: Exosome proteomes from normoxic hypoxic and gentamicin challenged SL pericites were investigated in physiological condition and in inner ear pathological conditions induced by hypoxia and ototoxic drug.

Poster Session F08 – Intercellular and Inter-Organismal Crosstalk
Chairs: Patricia Xander and Agnieszka Bronisz 5:15–6:30 p.m.

PF08.01

Human seminal plasma exosomes carry key proteins for spermatozoa capacitation

Valentina Murdica[1], Elisa Giacomini[2], Alessandra Alteri[2], Natasa Zarovni[3], Andrea Salonia[1], Paola Viganò[2] and Riccardo Vago[1]

[1]Urological Research Institute, IRCCS San Raffaele Hospital, Milano, Italy; [2]Reproductive Sciences Laboratory, Division of Genetics and Cell Biology, IRCCS San Raffaele Hospital, Milano, Italy; [3]Exosomics Siena SpA

Introduction: Male factor infertility is partially or fully responsible for up to half of infertility cases and, among them, a relevant share results from impairment of sperm maturation. Despite assisted reproductive technologies being increasingly used to cope with infertility, to date the fertilisation rate is only partially effective by employing apparently normal semen. Recently, a certain attention has been directed to the role of exosomes in spermatozoa maturation and in conferring overall fertilisation capacity by the transfer of key molecules along the male reproductive tract
Methods: We collectedseminal plasma from normozoospermic patients upon obtaining approval by the local ethical committee and informed consent by the patient. Exosomes were isolated and characterised by nanoparticle tracking analysis, transmission electron microscopy and western blot. The uptake of exosomes derived from seminal plasma and labelled with a fluorescent dye by spermatozoa was monitored by immunofluorescence.
Results: Seminal plasma contain both microvesicles and exosomes displaying canonical protein markers such as CD9, CD63, Alix and TSG101. In addition, exosomes, which represent a discrete population, carry proteins involved in the spermatozoa maturation and fertilisation capacity and in the mechanism of anti-oxidative protection. After ejaculation, sperm cells are still receptive and can continue to receive vesicle-delivered cargos. Indeed, we demonstrated that spermatozoa uptake exosomes derived from different sources.
Conclusion: Exosomes play a strategic role in sperm maturation and capacitation along the male reproductive tract, but also after ejaculation, opening new perspectives for the assisted *reproductive* technology.
Funding: The project was funded by intramural grant programme.

PF08.02

Novel multiparametric high resolution flow cytometry to sort cell-specific and size-specific extracellular vesicles

Terry K. Morgan[1] and Kevin Judge[2]

[1]OHSU; [2]BD Biosciences

Introduction: There is intense interest in developing new methods to perform liquid biopsies of tumours using blood samples. This is possible because tumours release millions of lipid encapsulated extracellular vesicles (EVs)/ml into the blood stream. The term EVs includes small exosomes (50–150 nm) and larger sub-micron sized microvesicles. Progress in the field has limited, however, by the lack of cell and size-specific rapid isolation methods. To address this issue, our group has developed a new multiparametric high resolution flow cytometry (HRFC) sorting method that can reliably identify, quantitate, and purify cell- and size-specific EVs from any tumour of interest.
Methods: Submicron-sized polystyrene beads (100, 160, 200, 240, 300, 500, 900 nm) were used as sizing and sorting efficiency controls. We used placental EVs present at high concentrations in maternal blood to validate the method and then began experiments testing pancreatic ductal adenocarcinoma specimens compared with negative controls. Sorted EVs of various sizes and from various cell types (e.g. placenta, platelets, pancreas) were characterised by electron microscopy, and used to test whether there was enrichment of target-specific protein and microRNA markers.
Results: Cell and size-specific EVs can be resolved and sorted to a high level of purity (>99%) using as little as 10 ul of plasma to generate 10^5 isolated EVs (10^7/ml) within 10 minutes. Sorted placental EVs are positive for exosome markers like CD9 and Annexins. They are positive for trophoblastic markers like placental alkaline phosphatase and placental-related microRNAs. Electron microscopy confirms sorted EVs are the expected size, purity, and concentration. CD41 positive platelet EVs are present in similar concentrations, but are a distinctly different size, ranging from 350–500 nm.
Conclusion: Using blood samples from pregnant women as a model for enriched "tumour" EV populations we have validated our new multiparametric HRFC sorting method. This novel technology provides a rapid means to characterise, count and isolate cell and size-specific EVs from patient plasma.

PF08.03

Extracellular vesicle-associated TIMP-1 and PAI-1 significantly enhanced pre-eclampsia predictive value of plasma placental growth factor in low risk population

Kok Hian Tan[1], Soon Sim Tan[2], Mor Jack Ng[1], Wan Shi Tey[1], Wei Kian Sim[2], John Carson Allen[3] and Sai Kiang Lim[2]

[1]KK Women's and Children's Hospital; [2]A*STAR; [3]Duke-NUS

Introduction: Circulating extracellular vesicles (EVs) such as cholera toxin B chain (CTB)- or annexin V (AV)-binding EVs were previously shown to be rich sources of biomarkers. Here we test if previously identified pre-eclampsia (PE) candidate biomarkers, TIMP-1 in CTB-EVs (CTB-TIMP) and PAI-1 in AV-EVs (AV-PAI) complement plasma PlGF in predicting PE in a low risk obstetric population.
Methods: 843 prospectively banked plasma samples collected at 28 + 0 to 32 + 0 gestation weeks in the Neonatal and Obstetrics Risk Assessment (NORA) cohort study were assayed by sandwich ELISAs for plasma PlGF, CTB-TIMP and AV-PAI. 19 patients subsequently developed PE 7.3 (±2.9) weeks later at a mean gestational age of 36.1 ± 3.5 weeks. The biomarkers were assessed for their predictive accuracy for PE using stepwise multivariate logistic regression analysis with Firth correction and areas under the curve (AUC).
Results: To achieve 100% sensitivity in predicting PE, the cut-off for plasma PlGF, CTB-TIMP1 and AV-PAI1 were set at less than 1235, less or equal to 300 or more than 1300, and more than 10,550 pg/mL plasma, respectively. The corresponding AUCs, specificity and PPV at 95% confidence interval were 0.92, 52.1% and 4.7%, 0.72, 44.5% and 4.0%, and 0.69, 21.5% and 2.9%, respectively. At 100% sensitivity, the three biomarkers had a combined AUC of 0.96, specificity of 78.6%, and PPV of 9.9% PPV
Conclusion: This is the first large cohort validation of the utility of EV-associated analytes as disease biomarkers. Specifically, EV biomarkers enhanced the predictive robustness of an existing PE biomarker sufficiently to justify PE screening in a low risk general obstetric population.

PF08.04

Identification of embryo competence by flow cytometric analysis of nucleic acid-containing MVs in embryo culture media

Éva Pállinger[1], Zoltán Bognár[2], József Bódis[3], Timea Csabai[2], Nelli Farkas[4], Krisztina Gödöny[3], Ákos Várnagy[3], Edit I Buzás[1] and Júlia Szekeres-Barthó[5]

[1]Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary; [2]Department of Medical Biology, Medical School, Pecs University, Pecs, Hungary; [3]Department of Obstetrics and Gynaecology, Medical School, Pecs University, Pecs, Hungary; [4]Department of Bioanalysis, Medical School, Pecs University, Pecs, Hungary; [5]MTA – PTE Human Reproduction Research Group

Introduction: Currently the efficiency of *in vitro* fertilisation (IVF) is about 30% in humans. To increase the chances of implantation, many IVF centres transfer more than one embryos, although multiple pregnancies are among the most common causes of preterm birth and pregnancy complications. According to this, it would be crucial to select the embryo that is most likely to implant and transfer that singular embryo only.
Methods: We developed a test, based on flow cytometric detemination of the nucleic acid containing (propidium iodide positive = PI+) extracellular vesicle (EV) count in day 5 embryo conditioned media.
Results: 88 women undergoing IVF were included in the study. In most cases more than one embryos were transferred. The intervention was successful in 58 cases, while implantation failure was detected in 30 patients. PI+ EV count was correlated with clinical outcome of pregnancy. The number of PI+ EVs was significantly lower in the "clinical pregnancy" group compared to the unsuccessful, "implantation failure" group. Cut off level of PI+ EV count was calculated by the analysis of the clinical outcome of single-embryo-transfer cases that resulted in a singleton pregnancy or, the two-embryos-transfers which eventuated twin pregnancies. The embryo culture media of these "confirmed competent" embryos contained lower level of PI+ EVs which suggests that a competent embryo can indeed be identified by low PI+ EV counts.
Conclusion: We developed a non-invasive, simple, cheap, rapid test, which identifies the embryos that are most likely to implant.

PF08.05

Embryo-endometrium cross-talk: characterisation of extracellular vesicles from in vitro cultured human embryos

Giacomini Elisa[1], Riccardo Vago[2], Ana Maria Sanchez[1], Paola Podini[3], Natasa Zarovni[4], Valentina Murdica[2], Roberta Rizzo[5], Daria Bortolotti[5], Jennifer Ovalle[1] and Paola Viganò[1]

[1]Reproductive Sciences Laboratory, Division of Genetics and Cell Biology, IRCCS San Raffaele Hospital, Milano, Italy; [2]Urological Research Institute, IRCCS San Raffaele Hospital, Milano, Italy; [3]Department of Neuroscience, Institute of Experimental Neurology, IRCCS San Raffaele Hospital, Milano, Italy; [4]Exosomics Siena SpA; [5]Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Italy

Introduction: Successful embryo implantation and consequent pregnancy is critically dependent on a two-way communication between the maternal uterus and the blastocyst. However, given the ethical restrictions and the lack of mechanistic studies, the identification of key embryonic signals remains so far elusive. There are plenty evidence on that extracellular vesicles (EVs) shuttled biomolecules can profoundly affect the phenotype and activity of their target cell and proofs of EV secretion have been reported in most cell types including embryonic stem cells and *in vitro* produced embryos derived from some mammalian species.
Methods: We collectedspent medium from embryo culture at day 3 and day 5 after fertilisation, upon ethical committee approval and informed consent. EVs were isolated and characterised by nanoparticle tracking analysis and transmission electron microscopy. The presence of specific EVs proteins and RNAs were investigated by western blot and RT-PCR. The uptake of EVs derived from embryos and labelled with a fluorescent dye by primary endometrial cell was monitored by immunofluorescence.
Results: Conditioned media from non-manipulated human embryos cultured *in vitro* for 3 days or up to the blastocyst stage contain EVs with a diameter of 30–300 nm and display traditional EV marker proteins CD63, CD9 and Alix. The embryonic origin of these EVs was confirmed by the presence of stemness gene transcripts (*NANOG* and *POU5F1*) and their enrichment in the non-classical HLA-G protein at appropriate stages of development, accordingly to their relative pattern in blastocysts. We also show the preferential uptake of dye-labelled embryo-derived EVs by primary endometrial cells.
Conclusion: Summary/conclusion: Our findings suggest EV exchange as an emerging way of communication at the maternal–foetal interface and raise some exciting possibilities regarding their potential therapeutic use as a co-factor for promoting the establishment of a successful pregnancy.
Funding: The project was funded by Merck Serono Grant For Innovation.

PF08.06

Human follicular fluid-derived exosome (folliculosome) non-coding RNA content is associated with ovarian reserve

Brandon A. Wyse[1], Shlomit Kenigsberg[1] and Clifford Librach[2]

[1]CReATe Fertility Centre; [2]CReATE Fertility Centre, Department of Obstetrics and Gynaecology, Department of Physiology, Institute of Medical Sciences, University of Toronto, Department of Gynecology, Women's College Hospital

Introduction: The ovarian follicle is the basic female reproductive unit containing the oocyte, somatic cells and follicular fluid (FF). Proper intercellular signalling between these compartments is required for optimal folliculogenesis, ovulation, and hormonal secretion.
Recent studies have explored human FF exosomes, also known as *folliculosomes* (FFEs). FFE miRNAs have been implicated as potential biomarkers for Polycystic Ovarian Syndrome (PCOS), blastocyst development, and pregnancy outcome. However, other classes of non-coding RNAs (ncRNAs) have yet to be characterised in FFEs.
Methods: This study was approved by the University of Toronto Ethics Board. FF was collected from individual follicles at ovum retrieval during *in vitro* fertilisation (IVF) procedures from consenting patients with normal, low, or high anti-Müllerian hormone levels (AMH), which is indicative of ovarian reserve ($n = 9$ patients). FFEs were isolated using the exoEasy kit (Qiagen). The number and size of particles was determined using NanoSight and the purity was confirmed by Western blotting. RNA was isolated using the NORGEN RNA isolation kit and sequenced using the IonTorrent platform. Bioinformatic analysis was conducted using Partek Flow.
Results: Several novel miRNAs were found to be differentially expressed in FFEs from patient subgroups. Comparing high vs. normal subgroups, miR125b, miR21 and miR22 were significantly downregulated by 4.6 fold ($p < 0.01$). We also observed significant downregulation of several miRNAs in FFEs that have previously been identified as potential biomarkers for PCOS and/or blastocyst development (miR30a and let7b). Several piwi protein-interacting RNAs (piRNA) were also identified. However, only two piRNAs (PIR36707 and PIR36741) were found to be differentially expressed between the 3 subgroups.
Conclusion: We identified several novel miRNAs that are differentially expressed between high, normal, and poor ovarian reserve subgroups. This is the first report identifying piRNAs in FFEs by small RNA sequencing. However, the biological significance of these piRNAs in folliculogenesis is unknown. These sncRNAs further expand our understanding of the complex communication network in the follicle and provide an opportunity for the development of novel biomarkers for oocyte quantity.

PF08.07

Plasma exosomes miRNAs profile and placental dimensions in the first trimester in gestational diabetes mellitus

Virginie Gillet, Larissa Takser and Annie Ouellet

Université de Sherbrooke, Canada

Introduction: Gestational diabetes mellitus (GDM), a common pregnancy complication, is related to placental dysfunction. Recent evidence show differential miRNAs expression between GDM pregnancies and uncomplicated pregnancies in the second and third trimester. Exosomes, nanovesicles of 30–100 nm, are released by placenta in maternal circulation and contained placental miRNAs. As well, it was noted that placental volumes are increased in second and third trimester in GDM pregnancies.
Methods: The aims of the study were to determine the expression profile of 15 selected miRNAs in plasma exosomes and to examine the association between maternal plasma exosomes-miRNAs expression and placental measurements in cases of GDM in comparison to uncomplicated pregnancies.
Results: Prospective case-control study nested in a cohort of pregnant women recruited before 14 weeks of gestation was conducted. 14

singleton pregnancies complicated by GDM and 15 singleton normal pregnancies were matched for gestational age. miRNAs were extracted from plasma exosomes (including placental exosomes) and their expression profile was determine by qRT-PCR. Placental maximal length and placental thickness were measured on the first-trimester ultrasound between 11–14 weeks of gestation.
Conclusion: We observed an overexpression of 7/15 miRNAs in GDM group compare to normal group. We reported a negative correlation between placental thickness and the expression of miR-122, miR-29a/b, miR-376c and miR-517 for pregnant women who later develop a GDM, but not for women with an uncomplicated pregnancy. Finally, a negative correlation were found between maximal placental length and expression of miR-1323, miR-136, miR-182, miR483 and miR-494 in controls groups but not in GDM group.CONCLUSION: Our data suggest that the expression of specific miRNAs released by trophoblast through exosomes in GDM and normal pregnancy is closely related to ultrasonographic placental measurements early in pregnancy. An inverse correlation between miRNAs expressions and placental dimensions in GDM could be the manifestation of an early dysregulation in placental metabolism due to the disease. Further studies are needed to explore the role of placental exosomes and miRNAs as potential early non-invasive indicator of placental abnormal development.

PF08.08

Role of the endogenous retroviral envelope glycoprotein Syncytin-2 in the uptake of placental exosomes by trophoblast and endothelial cells

Caroline Toudic[1], Xavier Elisseeff[1], Yong Xiao[1], Antoine Beaulieu[1], Adjimon Gatien Lokossou[2], Éric Rassart[1], Julie Lafond[1] and Benoît Barbeau[1]

[1]Université du Québec à Montréal, Centre de recherche BioMed, Montreal, Canada; [2]École polytechnique d'Abomey Calavi, Centre Hospitalier et Universitaire Mère et Enfant Lagune

Introduction: During pregnancy, the human placenta releases hormones, growth factors, cytokines and extracellular vesicles (EV) that modulate maternal physiology. Placental EV are released from the syncytiotrophoblast (STB), a multinucleated structure at the contact zone between maternal and foetal blood. Among EV, placental exosomes (Exo) are known to modulate the maternal immune system and remodel spiral arteries. Interestingly, the human endogenous retroviral protein Syncytin-2 (Syn-2), an important player of STB formation, is also found on local and circulating placental Exo. Our previous results showed that Syn-2 helps in the internalisation of placental Exo in trophoblast cells. We investigate here the role of Syn-2 in the entry of placental Exo in trophoblast and endothelial cells.
Methods: Exo were isolated from cell supernatants of Syn-2-expressing HEK293T and villous cytotrophoblasts (VCTB) using serial ultracentrifugation and characterised by TEM and NTA. Syn-2 was detected by western blot and flow cytometry. Exo were stained with the fluorescent dye PKH67 and their internalisation in VCTB, trophoblast-like BeWo and HUVEC endothelial cells was monitored by live cell imaging and flow cytometry.
Results: Flow cytometry confirmed the presence of Syn-2 on Exo from transfected HEK293T and VCTB cells. The incubation of placental Exo on VCTB, BeWo and HUVEC showed different internalisation rates but similar perinuclear region localisation. Brefeldin-A treatment (2 μg/ml) of HUVEC cells showed a 2-fold reduction in Exo internalisation compared to control, suggesting an endocytosis-dependent entry, as it was shown for BeWo and VCTB. The role of Syn-2 is now being assessed by comparing internalisation of Syn-2+ and Syn-2- Exo in trophoblast and endothelial cells.
Conclusion: Our data show that placental Exo are internalised in different cells in a similar manner. We are currently investigating the role of Syn-2 in this process and are further extending our analysis to exosomes derived from extravillous cytotrophoblast. In term, this work will provide new knowledge on the uptake of placental exosomes by trophoblast and endothelial cells and on the functional association between Syn-2 and placental exosomes.

PF08.09

Withdrawn at author's request.

PF08.10

Genetic content of EVs from fish pathogens

Petter Langlete and Hanne Winther-Larsen

University of Oslo, Oslo, Norway

Introduction: *Francisella noatunensis* is a severe threat to the worldwide fish farming industry, as there are currently no satisfactory vaccines available. The bacteria induce francisellosis, which is characterised by symptoms like discoloration, swollen kidney and spleen, formation of granulomas and high mortalities, especially in farming enclosures. The bacterium secretes extracellular vesicles (EVs), which have been proposed as vaccine candidates for the pathogens, but their protection is inconsistent and show varying levels of virulence. EVs from Gram-negative sources have been shown to carry DNA between bacteria, so there is significant reason to suspect that this is the case with *F. noatunensis* as well. Little is known about whether specific sequences of DNA are secreted in these EVs, or if the inclusion consists merely of random fragments of the genome or plasmids.This naturally raises the concern of how the use of EVs as vaccines could be affecting the spread of antibiotic resistance genes.
Methods: *F. noatunensis* is grown in 200–300 ml of rich growth media until a late exponential growth phase before vesicles are isolated by a centrifugation-based protocol. Ultra-thin (~45 nm) cryosections of fixed, DNAse-treated vesicle samples were imaged by transmission electron microscopy under the addition of immuno-gold-labeling to confirm the presence of DNA within the membranes.
Results: Vesicles are DNase treated, lysed and the DNA is isolated for multiple displacement amplification (MDA) and bioanalyzer inspection, to map concentrations and fragment lengths of present DNA. Depending on concentration, a protocol for obtaining enough vesicle-associated DNA will be constructed.
Conclusion: Immuno-gold electron microscopy confirmed the presence of DNA inside vesicles, and this was also confirmed by MDA.
Summary: This project aims to investigate the DNA and RNA content of extracellular vesicles from fish pathogens such as Francisella and Piscirickettsia. So far in the project, I have confirmed the presence of DNA inside the vesicles, and an analysis on DNA concentration and fragment length distribution is in progress. After this is known, we construct a protocol for quantitative sequencing on RNA and DNA to assess preferential packing.

PF08.11

Orange juice contain two types of extracellular vesicles with biological activities

Pascal Colosetti[1], Emmanuelle Berger[2], Alain Geloen[3] and Sophie Rome[2]

[1]UMR Inserm U1060/INRA 1397; [2]INRA; [3]CNRS

Introduction: Our project is based on the concept that edible plants contain extracellular vesicles (EVs) that may be used to restore muscle homeostasis in the context of metabolic diseases. In this study we have isolated and characterised EVs from juice of *Citrus sinensis* (L.) Osbeck and have tested their activity on muscle cell proliferation.
Methods: Orange juice from untreated fruits was obtained by hand-pressing, centrifugated at 3000*g* for 30 min, at 10,000*g* for 60 min, filtered at 0.45 mm, centrifugated at 16,500*g* for 60 min and ultracentrifugated at 100,000*g* for 90 min. The resulting pellet was further purified on sucrose/D_2O gradiant or with qEVoriginal size exclusion columns of approximately 75 nm pore size (iZON). Protein concentrations and AChE activity were measured on each fraction. EVs were labelled with PKH27 and their captation was analysed on recipient cells. Their effect on cell proliferation was tested on C2C12 muscle cells by using the xCELLigence real time cell analysis system (Roche Applied Science).

Results: EVs from the ultracentrifugation pellet (EVs-UC) modulated proliferation, ROS production and triglyceride synthesis of C2C12. EVs-UC were incorporated into intestinal CaCo-2 cells within 1 h. Analyses with TEM and NTA showed that EVs-UC is composed of 2 types of EVs of different sizes and morphologies. The small EVs-UC (SEVs-UC) were spheric and homogenous in size (150 nm). Interestingly, SEVs-UC were excluded before LEVs-UC from iZON columns suggesting a possible interaction with the resin carbohydrates. Sucrose/D_2O gradient of EVs-UC subdivided LEVs-UC into two populations of different densities and colours (orange and white). Only LEVs-UC from the white ring had AChE activity. SEVs-UC were found in the sucrose/D_2O gradient pellet indicating that they have higher density than the 2 LEVs-UC subpopulations. Moreover only SEVs-UC repressed C2C12 proliferation.
Conclusion: These preliminary results indicate that EVs from orange juice might have interesting properties to restore muscle homeostasis during metabolic diseases and could participate in health benefits of long-term orange juice consumption such as cardiovascular protection.

LBP.26

Porcine *in vitro* maturation co-cultured with different donor age of human adipose derived stem cell followed by parthenogenetic activation

Erif Maha Nugraha Setyawan, Min Jung Kim, Hyun Ju Oh, Geon A Kim, Seok Hee Lee, Jun-Xue Jin and Byeong Chun Lee

Seoul National University, Seoul, Republic of Korea

Introduction: Research efforts are increasing focused on extracellular vesicles (EVs) as novel mediators of intercellular communication. EVs are membrane-bound vesicles released by every cell type that has been studied to date. Adipose derived stem cells (ASCs) secrete EVs which have roles on oocyte maturation. The aim of this study was to determine the porcine embryo development after maturated under co-culture system with different donor age of ASCs.
Methods: The human ASCs (derived from young 1,2 (Y1 and Y2) and old 1,2 (O1 and O2)) were seeded $1x10^5$ cells into 12-wells plate with AMSC medium and incubated in a 37 °C, 5% CO_2 incubator for 24 h. After reached 70% confluence, the media was changed to IVM media and COCs were incubated with trans-well in first 22 h using hormone and the second 22 h without hormone. Embryo developments such as cleavage rate, blastocyst rate and blastocyst cell number were analyzed using ANOVA continued with Duncan test in SPSS.
Results: Oocyte maturation and cleavage rates were significantly increased in Y2 and O2 (88.0 and 86.3%, 83.2 and 83.5%, respectively) than other groups (74.0, 78.5 and 75.0%, 67.0, 63.5 and 62.0%, respectively). The ASCs co-culture with Y1 and Y2 groups showed higher percentage of blastocyst rate compared to control group (23.8 and 23.0 vs. 16.1%, respectively, $P < 0.05$). Total cell number in all ASCs co-culture groups exhibited higher number of cells compared with control group.
Summary/Conclusion: Although oocyte maturation and cleavage rates have variation with different human ASCs, the blastocyst formation rate was increased in young human ACSs. There is a critical need for further functional and mechanistic studies to provide conclusive experimental evidence in intercellular communication mediated by EVs which might be contributed to oocyte and embryo development.
Funding: This study was supported by Korea IPET (#114059-03-3-SB010), Nature Cell (#550-20150030), Research Institute for Veterinary Science, and the BK21 plus program.

Poster Session F09 – EVs in Parasitic Diseases
Chairs: Amy Buck and Rodrigo Soares 5:15–6:30 p.m.

PF09.01

Impact of GP63 enrichment in Leishmania-derived exosomes in the development of cutaneous leishmaniasis

Alonso da Silva Lira Filho and Martin Olivier

McGill University, Montreal, Canada

Protozoan parasites of the genus Leishmania are transmitted by the bite of infected sand flies leading to a wide-range of diseases called leishmaniasis. Depending on the species involved, it can produce a self-healing wound to a potentially lethal visceral infection. Recently, we published a seminal work demonstrating that leishmanial exosomes (Leish Exo) were released in the lumen of the sand fly midgut and to be co-egested with the parasite during the blood meal. Leish Exo were found to stimulate an inflammatory response conducting to exacerbated cutaneous leishmaniasis, also it was shown that these vesicles cargo important virulence factors like GP63, a metalloprotease that regulate many important macrophage functions. First, we have been interested to identify the immune sensors/receptors triggered by Leish Exo leading to the skin hyperinflammatory response. Second, we wanted to analyse the impact of GP63 in Leish Exo on the modulation of macrophage inflammatory response and its infection in mice. C57BL/6 knockout mice were used in the screening of receptors involved in the recognition of either single or double stranded RNA, DNA, peptides or lipids enriched in these vesicles, having their footpad infected with stationary Leishmania major with or without Leish Exo. Additionally, using Leish Exo isolated from *L. amazonensis* expressing different amounts of GP63 (WT, GP63low, GP63high) we tested their capacity to induce the expression of various cytokines (IL-1, TNF, IL-6) and chemokines (CCL2) on cultured macrophages. Finally, we infected Balbc mice in their footpad with stationary *L. amazonensis* without or with Leish Exo from each three groups of parasites. Results obtained revealed that specific sensors are involved in recognition of Leish Exo and that GP63 enrichment in these vesicles induced differential modulation of macrophage responses correlating with a distinctive skin hyperinflammatory responses. Further information and discussion will be provided during the poster session. This work was funded by a CIHR grant.

PF09.02

Characterisation of extracellular vesicles released by *Leishmania amazonensis* and its role on macrophages activation

Fernanda MC. Barbosa[1], Mayte dos S. Toledo[1], Talita V. Dupin[1], Kleber S. Ribeiro[2], Andre Cronemberger-Andrade[3], Alison FA. Chaves[2], Ana Cláudia Torrecilhas[4] and Patricia X. Batista[5]

[1]Universidade Federal de São Paulo campus Diadema, Sao Paulo, Brazil; [2]Universidade Federal de São Paulo, Sao Paulo, Brazil; [3]UNIFESP; [4]Universidade Federal de São Paulo – UNIFESP, Sao Paulo, Brazil; [5]Departamento de Ciências Farmacêuticas, Universidade Federal de São Paulo campus Diadema, Sao Paulo, Brazil

Extracellular vesicles (EVs) are released by many pathogens. These EVs perform several functions, such as delivering molecules that perform effector activity on host cells. Some studies have demonstrated that EVs released by some species of Leishmania appear to contribute to the establishment of infection and immunomodulation. However, studies have not been performed to verify the role of EVs produced by *L. amazonensis* (specie responsible for cutaneous leishmaniasis in Brazil) in the activation and/or modulation of phagocytic cells of the immune system and disease progression. This work aimed to characterise the EVs released by *L. amazonensis* promastigotes and its influence on macrophage activation. We showed by nanoparticle tracking analysis and scanning electron microscopy that *L. amazonensis* promastigotes spontaneously released EVs at different time (1, 2, 4 and 24 h) and at several temperatures (26, 34 and 37°C). These EVs modulated the medullary macrophages response. It was also observed a significant reduction in infection of bone marrow macrophages stimulated with vesicles compared to unstimulated bone marrow macrophages. A different cytokines profile was observed in stimulated macrophages as compared to untreated cells. This data can contribute to a better understanding of the host-parasite relationship and modulation/activation of the immune system in *L. amazonensis* infection. These results can be further used in the identification of new molecular targets as well as the development of alternative therapeutic and diagnostic strategies.

PF09.03

B-1 cells infected with *Leishmania amazonensis* promastigotes release extracellular vesicles that act as a novel mediator of macrophages activation

Mayte dos S. Toledo[1], Fernanda MC. Barbosa[1], Andre Cronemberger-Andrade[2], Natasha FC. Reis[1], Ana Cláudia Torrecilhas[3] and Patricia X. Batista[4]

[1]Universidade Federal de São Paulo campus Diadema, Sao Paulo, Brazil; [2]UNIFESP; [3]Universidade Federal de São Paulo – UNIFESP, Sao Paulo, Brazil; [4]Departamento de Ciências Farmacêuticas, Universidade Federal de São Paulo campus Diadema, Sao Paulo, Brazil

Immune cells can release different type of extracellular vesicles (EVs) that are relevant vehicles of intercellular communication. EVs targeted various types of immune cells and are involved in immune regulation depending on the context. B-1 cells are a subtype of B lymphocytes with peculiar functions in immunity. These cells are able to produce regulatory cytokines (mainly IL-10), natural antibodies and differentiate into phagocytic cells. In this study we evaluated the ability of B-1 cells in producing extracellular vesicles in the presence or absence of *L. amazonensis* promastigotes and their influence on macrophages activation. Our results showed that B-1 cells spontaneously released EVs but there were increase in releasing after 24 or 48 h of *in vitro* infection, as demonstrated by nanoparticle tracking analysis and scanning electron microscopy. Macrophages from BALB/c mice treated with EVs from infected B-1 cells led to a significant increase in IL-6 and IL-10, as compared to the cells stimulated with EVs released by non-infected B-1 cells. No differences were observed to TNF-alpha and iNOS. These macrophages did not alter the phagocytic index (PI) after treatment with EVs from infected or non-infected B-1 cells. To macrophages from C57BL/6 mice we observed a significant reduction in the expression of IL-10 and *iNOS* but the expression of IL-6 and TNF-alpha were increased in macrophages stimulated with EVs from infected B-1 cells, as compared to macrophages stimulated with EVs released by non-infected B-1 cells. Moreover, these macrophages treated with EVs from infected B-1 cells have a significant increase in the phagocytic index as compared to the same cells stimulated with EVs from non-infected B-1 cells. Our work showed that B-1 cells are able to release EVs but the infection stimulated an increase in their production. These EVs modulated the expression of some cytokines and iNOS on macrophages and led an increase in PI in macrophages from C57BL/6 mice.

PF09.04

Unravelling the exosome pathway in the human pathogen leishmania

Vanessa Diniz Atayde and Martin Olivier

McGill University, Montreal, Canada

Leishmania are ancient unicellular eukaryotes specialised in the infection of macrophages. They cause a spectrum of diseases, in which severity is related to the presence of numerous parasitic virulence factors that are capable of triggering diverse inflammatory outcomes in the host. Recently, we demonstrated that Leishmania exosomes are virulence factors, as they are transmitted to the host during the sand fly bite alongside the parasite and exert an important role in the establishment of the disease. Although we have a quite good understanding on the role of Leishmania exosomes during infection, little is known about their biogenesis and secretion by

the parasite. In higher eukaryotes, the exosome pathway has been well described. Efforts in finding exosome-specific markers have permitted their characterisation as a unique population and have further confirmed their biogenesis mechanisms. Proteomics studies are especially useful for these means, since they catalogue vesicle content, which may hint at their intracellular origin. Here, we analysed Leishmania exosome content by mass spectrometry, using bioinformatics tools to fish out Leishmania orthologues of described mammalian exosome-enriched proteins. We found that Leishmania exosomes contain significant amounts of EHD4 and Annexin XI markers, as well as molecules involved in the exosome pathway such as VPSs, Alix, Radixin and Rab11. In order to validate these findings, we are currently in the process of knocking down some of these proteins, to access their impact on exosome secretion and hence parasite virulence. This work is relevant for its potential in finding new drug targets to treat severe leishmaniasis and for unravelling Leishmania exosome biomarkers for diagnostics.

PF09.05

The protozoan parasite *Trypanosoma cruzi* viability is required for the release of extracellular vesicles

Camilla Ioshida[1], Rodrigo Soares[2], Andre Cronemberger-Andrade[1] and Ana Cláudia Torrecilhas[3]

[1]UNIFESP; [2]René Rachou Research Centre, Brazil, FIOCRUZ; [3]Universidade Federal de São Paulo – UNIFESP, Sao Paulo, Brazil

Introduction: *Trypanosoma cruzi* is a flagellated protozoan that causes Chagas' disease. It circulates in the bloodstream as trypomastigotes, which invade several mammalian cell to proliferate as amastigotes. Trypomastigotes hatched from infected mammalian cells in culture were found to release EVs that modulate infectivity in the mammalian host. Parasite EVs contain the major surface components of the parasite and their release depends on the parasite strain. However, it is unknown the mechanism of EVs release and whether it occurs as a consequence of parasite damaging. Here we investigated EVs release in conditions that affect parasite viability.
Methods: Trypomastigotes were collected from infected mammalian cells and incubated for 2 h under different conditions. After the incubation, parasites were tested for viability using Presto Blue Reagent. Vesiculation was observed by scanning electron microscopy. EVs were isolated by size exclusion chromatography (SEC) and characterised by nanoparticle tracking analysis (NTA).
Results: The amount and size of EVs was similar from 4°C to 37°C, conditions that did not affect parasite viability. In contrast, an increase in size and decrease in concentration of the EVs were observed when trypomastigotes were incubated with 0.01% of NaN_3 with a parallel decrease in the cellular viability. Maximal release was observed between pH 5 and 7. Outside this range the release was reduced, with a simultaneous decrease in viability with visible changes in the parasite morphology. Oxidative agents such as $NaNO_2$ also affected EVs release at conditions that cell viability was reduced.
Conclusion: We conclude that parasite viability and/or integrity is required by EVs release.

PF09.06

Extracellular vesicles derived from heligmosomoides polygyrus represent a novel target for vaccine-induced immunity

Gillian Coakley[1], Jana L. McCaskill[2], Jessica G. Borger[3], Henry J. McSorley[4], Amy H. Buck[2] and Rick M. Maizels[1]

[1]Wellcome Centre For Molecular Parasitology, Institute for Infection, Immunity and Inflammation, University of Glasgow, United Kingdom; [2]Institute of Immunology and Infection Research, Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, United Kingdom; [3]The Peter MacCallum Cancer Centre, Melbourne, Australia; [4]MRC Centre for Inflammation Research, The Queens Medical Research Institute, University of Edinburgh, United Kingdom

Introduction: Extracellular vesicles (EVs), including exosomes, facilitate cellular communication through the transfer of small RNAs, lipids and proteins. It has been shown that parasites secrete EVs which can play a key role in both pathogenicity and host immunoregulation, and that parasite-derived EVs directly modulate the host immune response. In particular, we demonstrate that secreted vesicles from the murine gastrointestinal nematode *Heligmosomoides polygyrus* interfere with epithelial cell and macrophage innate responses to infection, inhibiting the type 2 immune response in the host that is required for parasite expulsion.
Methods: Comparative studies between mammalian and *H. polygyrus*-derived EVs highlight some of the key factors responsible for EV uptake, and showed that specific antibodies against parasite EVs interfere with their entry into mammalian cells *in vitro*, inhibiting any parasite-mediated effects on the host cell. Additionally, immunisation of mice using an EV/alum conjugate contributes to significant protection from a subsequent *H. polygyrus* infection. Immunity against larval challenge is seen through the initiation of specific antibody responses against EVs, and results in a substantial reduction of parasitic egg counts and adult worm burden. Using cross-link immunoprecipitation and mass spectrometry, we have identified the major candidate proteins from these EVs that are recognised by antibodies generated by the EV/alum vaccination schedule. Identification of these candidates has prompted further investigation into both the individual roles of these proteins during infection, and whether they serve as appropriate targets for vaccination against a subsequent *H. polygyrus* infection.
Conclusion: This work suggests that EVs secreted by nematodes could mediate the transfer and uptake of parasitic products into host cells, establishing cross-species communication to suppress the host immunity. Furthermore, gaining a better understanding of the molecular complexity of these EVs, and how they drive host immunity, will be crucial for the development of an efficient vaccine against nematode infection.

PF09.07

Extracellular vesicles releades by strains of *Leishmania enriettii* with different degrees of pathogenicity: extraction, purification and preliminary characterisation

Larissa Paranaiba[1], Armando Menezes-Neto[2], Ana Cláudia Torrecilhas[3] and Rodrigo Soares[2]

[1]Universidade Federal de Minas Gerais; [2]René Rachou Research Centre, Brazil, FIOCRUZ; [3]Universidade Federal de São Paulo – UNIFESP, Sao Paulo, Brazil

Introduction: *Leishmania enriettii* is a non-infectious species to man, whose reservoir is the guinea pig *Cavia porcellus*. Many aspects of the parasite-host interaction were studied involving its main surface glycoconjugates including lipophosphoglycans (LPGs) and glycoinositolphospholipids (GIPLs). Those glycoconjugates were important for immunopathogenicity via TLR2 and TLR4. Since those structures could be also present in the extracellular vesicles (EVs) of the protozoa. This project aims to investigate the role of those structures in *L. enriettii* pathogenicity.
Methods: Two strains (L88 and Cobaia), with different degrees of immunopathology were studied. Parasites were grown until stationary phase and vesicle release was stimulated by raising temperature (34–37° C) for 2–4 h in serum-free conditions. Supernatants were processed by differential centrifugation and vesicles were isolated by ultracentrifugation (100,000*g*) or size exclusion chromatography (SEC). Vesiculation was observed by electron microscopy and vesicles were quantitated by nanoparticle tracking analysis (NTA).
Results: Both strains were able to shed vesicles, as demonstrated by SEM analysis. NTA quantitatively confirmed SEM data. The EVs released by L88 and Cobaia strains had similar size distributions with modal sizes of 136 (±1.5) nm and 141 (±5.4) nm, respectively. After normalisation by cultured parasite concentrations, a slightly higher amount of EVs was observed for the cobaia strain, although this finding needs further confirmation. EVs from the Cobaia strain were isolated by size exclusion chromatography (SEC) and fractions were analysed by NTA and dot-blot for the detection of gp63, an important EV surface marker in Leishmania.
Conclusions: The detection of gp63 in the same fractions in which particles were detected by NTA, not only corroborates their vesicular nature but also suggests that *L. enriettii* EVs are likely to be involved in the immunomodulation of host cells. Further characterisation will be performed to qualitatively compare the EVs from both strains especially regarding their contents and funcionality in macrophages.Those features could help to understand the differences in their immunopathology.

PF09.08

Leishmania-derived extracellular vesicles express lipophosphoglycan (LPG) on their surface

Armando Menezes-Neto[1], Blima Fux[2], Frederic Frezárd[3], Valéria Borges[4], Albert Descoteaux[5], Ana Cláudia Torrecilhas[6] and Rodrigo Soares[1]

[1]René Rachou Research Centre, FIOCRUZ, Brazil; [2]Federal University of Espírito Santo (UFES); [3]Federal University of Minas Gerais (UFMG); [4]Gonçalo Moniz Research Centre, FIOCRUZ, Brazil; [5]Centre INRS–Institut Armand-Frappier; [6]Universidade Federal de São Paulo – UNIFESP, Sao Paulo, Brazil

Promastigote forms of Leishmania release exosomes that modulate microbicidal activities of macrophages. Previous studies have demonstrated that GP63 is an exosome surface marker for Leishmania. In the parasite surface, along with GP63, Lipophosphoglycan (LPG) is the major surface glycoconjugate and a multi-virulence factor. Among many functions, LPG modulates nitric oxide and cytokine production via TLR2 and TLR4 and inhibits phagolysosome biogenesis and oxidative burst. However, its presence in parasite-derived vesicles is yet to be demonstrated. We hypothesise that LPG is present in parasite vesicles and is involved in communication between parasite and host cell. Four strains (*Leishmania braziliensis* M2903, *L. infantum* BH46, *L. infantum* Ba262 and *L. infantum* Ba262-KO(lpg1-/-)) were grown to stationary phase and vesicle release was stimulated by raising temperature (34–37°C) for 2–4 h in serum-free conditions. Supernatants were processed by differential centrifugation and vesicles were isolated by ultracentrifugation (100,000*g*) or size exclusion chromatography (SEC). Vesiculation was observed by electron microscopy and vesicles were quantitated by NTA and Bradford assay. All strains produced vesicles in the expected size range of exosomes. Immunodetection of glycoconjugates was performed in ELISA or dot-blot assays, using an mAb anti-GP63 and the mAb CA7AE, specific for the Gal-Man-P epitope, present in LPGs of most Leishmania. LPG and GP63 were detected in the EV-containing SEC fractions. Our data strongly support that LPG is present in promastigote-derived EVs. Molecular shaving of EVs with proteinase K suggested that proteophosphoglycans (PPGs) might also be present. The immunomodulatory properties of these vesicles, specially the role of LPG, are being further investigated. These findings expand current knowledge on glycobiology of Leishmania and imply the applicability of vesicles as diagnostic or therapeutic tools against leishmaniasis.

Poster Session F10 – EVs as Mediators of Cancer Cell Signaling
Chairs: Ryan Pink and Valbona Luga 5:15–6:30 p.m.

PF10.01

A novel role for extracellular Hsp90 in exosome traffic from cancer cells

Daniel Wong, Aaron Bernstein, Kofi Gyan and Dan Jay

Tufts University School of Medicine, MA, USA

We and others identified extracellular Hsp90 (eHsp90) as pro-invasive using a FALI-based screen for proteins required for cancer invasiveness and showed that Hsp90 activates Matrix Metalloproteinase-2 (MMP2) to facilitate breast cancer invasion. Since then, many pro-invasive proteins activated by eHsp90 for different cancers have been identified by several labs suggesting that eHsp90 may serve as an activation hub for cancer invasion. Hsp90 is released from cancer cells via exosomes and appear on their outer surface and these exosomes enhance invasion in an Hsp-90 dependent manner. Together, these observations suggest that eHsp90 may act in exosome trafficking. We tested this notion by treating MDA-MB-231 breast cancer cells in culture with the Hsp90 inhibitors ganetespib or STA-12-7191 (a biotinylated and thus impermeant derivative of ganetespib) and measured the release of exosomes from these cells and the uptake of exosomes by stromal cells. Drug treatments resulted in a 50% reduction in exosome release as assessed by protein concentration and Hsp90 compared to no drug controls and markedly inhibited uptake of eHsp90-containing exosomes by stromal cells. The general mechanisms of exosome trafficking are poorly understood and we are examining and quantitating subpopulations of exosomes from drug-treated cells to address this. Thus, we have evidence that eHsp90 has roles in both exosome release and uptake by stromal cells, important processes for the communication of tumours with their extracellular milieu that enhance invasion.

PF10.02

Proteomic profiling of extracellular vesicles reveals differences in glucose metabolism reflecting cancer invasiveness

Steven G. Griffiths[1], Félix Royo[2], Juan M. Falcón-Pérez[2] and Alan A. Doucette[3]

[1]X0S0ME; [2]CIC bioGUNE; [3]Dalhousie University, Halifax, Canada

Introduction: Cancer cells universally exhibit dysregulated metabolism such as accelerated aerobic glycolysis, glutaminolysis and heme biosynthesis. These provide energy currency and precursors for new cells and exosomes as well as reducing power and redox control for rapidly proliferating cells. Lethality of a cancer and management options are determined by the degree and combination of these proclivities. We tested the hypothesis that vesicle-bound metabolic enzymes exported from breast cancer cell lines could differentiate between invasive, minimally invasive and non-transformed phenotypes.
Methods: EVs were recovered from bioreactor culture media by peptide affinity (*Venceremin, Vn96*) to heat shock proteins (HSPs) common to the surface of all cancer cells. Three breast cancer cell lines of varied tumorigenic phenotype were examined: invasive (SKBR3), ductal carcinoma (MCF7) and non-transformed (MCF10). EV proteins were resolved with a solution-based procedure GELFrEE. Discrete molecular weight ranges were subject to proteomic analysis *via* liquid chromatography/mass spectrometry (LC/MS).
Results: Enzymes typical of altered metabolic pathways were abundant in EVs from cancer cells. Ingenuity pathway analysis indicated that glycolysis/gluconeogenesis and the pentose phosphate pathway were at the top of 14 canonical pathways represented in SKBR3, second and fourth for MCF7 and absent from MCF10.
Conclusion: Invasiveness and stromal subjugation by cancer may be dependent upon the export of pleiotropic enzymes that disseminate the metabolic phenotype directly or through epigenetic influence among localised subclones and surrounding cells. EVs may also compartmentalise pathways from inhibitors. HSP affinity capture of EVs combined with GELFrEE provides a rich source of proteomic information to determine invasive capability of cell cultures with potential for use in screening EVs from liquid biopsy samples such as ascites, plasma or urine for staging and treatment options.

PF10.03

Effect of hypoxia on the exosome release and migration activity of a panel of ovarian tumour cell lines that mimic different stages of the tumour

Mona Alharbi[1], Shayna Sharma[1], Carlos Palma[1], Richard Kline[2], Katrina Wade[2], Jacob Estes[2], John Hooper[3], Gregory Rice[1] and Carlos Salomon[1]

[1]Exosome Biology Laboratory, Centre for Clinical Diagnostics, University of Queensland Centre for Clinical Research, Royal Brisbane and Women's Hospital, The University of Queensland, Brisbane, Australia; [2]Department of Obstetrics and Gynecology, Ochsner Baptist Hospital, New Orleans, LA, USA; [3]Mater Research Institute, University of Queensland, Translational Research Institute, Woolloongabba, Australia

Introduction: The aim of this study was to determine the effect of hypoxia on the exosomes release and migration capacity of nine ovarian tumour cell lines.
Methods: Ovarian tumours cell lines (i.e TOV-122, HEY, OVCAR429, SKOV-3, CAOV-3, OVCA420, A2780, OV-90 and OVCAR-3) and mesothelial cell line (MET-5A) were used in this study. Cells were cultured under 21% O_2 (normoxia) and 1% O_2 (hypoxia) for 48 h. Small extracellular vesicles were enriched from cell-conditioned media by differential centrifugation from 800*g*, 2000*g* and 12,000–100,000*g*. Exosomes were further enriched using 200 nm filter and ultrafiltration (100 kDa cutoff). Exosomes were characterised by electron microscopy and western blot (CD63 and TSG101), and quantified by nanoparticle tracking analysis (NanoSight). Migration capacity of cells was determined using a real-time imaging system (Incucyte).
Results: Exosomes were identified as spherical vesicles, with a typical cup or spherical-shape and diameters around of 100 nm and positive for CD63 and TSG101. Hypoxia increased the exosomes release ~3.5, ~7.3, ~3.0, ~2.5, ~3.0, ~13.2, ~2.4 and ~1.2-fold in TOV-122, OVCAR429, SKOV-3, CAOV-3, MET-5A, OVCA420, A2780 and OVCAR-3, respectively. Finally, the exosome release was positively correlated with migration capacity of cells.
Conclusion: This study established that hypoxia increase the exosome release in a wide range of ovarian cancer cell lines. Interestingly, exosome release was associated with the migration capacity of corresponding cells. Therefore, we suggest that exosomes concentration may be an indicator of tumour stage and invasiveness.

PF10.04

Impact of the oncogenic C19MC microRNA cluster on the vesiculation of human paediatric embryonal brain tumour cells- ETMR as a paradigm

Esterina D'Asti, Laura Montermini, Andrea Bajic, Nada Jabado and Janusz Rak

The Research Institute of the McGill University Health Center, Montreal, Canada

Introduction: Disorganised intercellular communication due to deregulated genetic and epigenetic molecular control represents a hallmark of paediatric embryonal brain tumours. Embryonal tumour with multilayered rosettes (ETMR) represents a paradigm of these events due to oncogenic amplification of the C19MC cluster, which drives widespread epigenetic deregulation of gene expression, a highly malignant phenotype as well as enrichment in cancer cell stemness. Since oncogenic

mutations often impact vesiculation and its related intercellular communication pathways, we explored the impact of C19MC and one of its key components, miR-520g, on the vesiculation of embryonal brain tumour cells.
Methods: ETMR cells (BT183) and embryonal brain tumour cells engineered to express miR-520g (DAOY and UW228) were tested for overall vesiculation, cellular RNA expression of vesiculation-related markers, and the proteome of extracellular vesicles (EVs) as a function of oncomir activity.
Results: We observed that miR-520g upregulates EV emission while changing the expression of genes involved in EV biogenesis (vesiculome) and impacting EV cargo (e.g. by suppressing the vascular regulatory protein known as tissue factor- TF). We verified the causality of miR-520g in this context and described the related changes in the EV proteome and RNA content, especially the levels of miR-520g itself. EVs from brain tumour cells harbouring miR-520g were tested for their effects on endothelial cell behaviour as ETMR exhibits highly haemorrhagic morphology.
Conclusion: Oncogenic microRNA associated with ETMR alters cancer cell vesiculation pathways in ways that may affect cell-cell communication and disease biology.

PF10.05

Impact of WNT signalling on the vesiculation of human medulloblastoma cells

Esterina D'Asti, Laura Montermini and Janusz Rak

The Research Institute of the McGill University Health Center, Montreal, Canada

Introduction: The WNT signalling pathway regulates intercellular communication, morphogenesis, and stemness in health and disease and is frequently overactivated in human brain tumours such as medulloblastoma (MB). This activation state is either ligand-dependent or results from gain-of-function mutations affecting beta-catenin (CTNNB1), the key mediator of WNT-regulated gene expression and a known oncogene. Since oncogenic lesions often exert their biological effects, at least in part, through their impact on the formation, cargo, and function of extracellular vesicles (EVs), we asked whether this pathway is affected by overactivation of WNT in MB cells in culture.
Methods: MB cell lines (DAOY and D283), were stimulated using soluble WNT3A ligand and characterised for EV emission as well as the RNA and protein expression profile of vesiculation markers.
Results: We observed multiple changes in cellular RNA encoding EV-regulating genes (vesiculome) in MB cells treated with WNT3A, along with changes in EV emission characteristics and protein cargo. Notably, a robust and consistent WNT3A-dependent downregulation of exosomal markers (CD63 and CD81) was noted in the total EV fraction by western blotting.
Conclusion: Activation of canonical WNT signalling may reduce and reprogram exosomal release from brain tumour cells. The significance of this finding in the context of MB biology is under study.

PF10.06

Detection, characterisation and function of extracellular vesicles in resistant melanoma

Giulia Cesi[1], Demetra Philippidou[1], Francois Bernardin[2], Yeoun Jin[2], Guillaume Van Niel[3] and Stephanie Kreis[1]

[1]University of Luxembuourg, Luxembourg; [2]Luxembourg Institute of Health; [3]Institut Curie, PSL Research University, Paris, France, CNRS

Introduction: Extracellular vesicles (EVs) are nano-sized structures that are released by all cell types under both physiological and pathological conditions. As EVs can be released by "donor" cells and taken up by "recipient" cells, they can be regarded as vehicles of intercellular communication or "homing pigeons" influencing key biological functions by delivering and transporting cytokines, growth factors, proteins, mRNAs and microRNAs. Recently, EVs have also been identified as new messengers in transferring drug resistance to still sensitive cells. In melanoma patients, drug resistance is a pressing issue. Despite the promising initial results obtained with vemurafenib and dabrafenib (BRAF kinase inhibitors) in the clinic, it soon became evident that these molecules were not able to provide durable responses, as resistance to treatment soon develops within months in almost all patients.
Methods: The content of the EVs released by sensitive melanoma cells and their corresponding drug resistant cells has been analysed by mass spectrometry. In addition co-culture experiments have been performed to understand the potential involvement of EVs in the "spreading" of drug resistance.
Results: We could show that sensitive melanoma cells acquire the drug resistant phenotype if co-cultured with EVs released by resistant cells. Proteomic analysis revealed different content profiles with a panel of proteins especially enriched in the "resistant extracellular vesicles". Hence, potential candidates that might play a role in conferring drug resistance have been identified.
Conclusions: Our results suggest that "resistant extracellular vesicles" have functional properties capable of making sensitive melanoma cells more resistant to BRAF inhibitors.

PF10.07

Mutant BRAF inhibition changes the expression of exosomal coding and non-coding RNAs released by melanoma cells

Taral Lunavat[1], Lesley Cheng[2], Robyn A. Sharples[2], Cecilia Lässer[3], Andrew F. Hill[2] and Jan Lötvall[1]

[1]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden; [2]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Victoria, Australia

Introduction: In melanoma, more than 50% of patients harbour BRAF mutations, most commonly the valine-600 (V600) variant. Vemurafenib is a BRAF-V600 inhibitor used for the treatment of late stage melanoma. Here we determine the effects of vemurafenib on melanoma cells and the RNA content in cells and exosomes.
Methods: Exosomes were isolated using a differential centrifugation protocol, followed by ion torrent sequencing to identify coding and non-coding RNAs. Sequences were aligned to the human genome (HG19) using the Torrent Suite, and files were transferred to Partek Genomic Suite and Flow (Partek Incorporated, Singapore) for mapping against miRBase V.21 and Ensembl Release 75 to identify miRNA, ncRNA and coding RNA species. Characterisation of exosomal proteins were validated by using Western blot.
Results: Inhibition of BRAF mutant melanoma cells with vemurafenib significantly altered the RNA contents in cells as well as in the exosomes. Exosomes from the treated cells showed differentially expressed miRNAs compared to the exosomes from the nontreated cells. Interestingly, hierarchical clustering of coding and non-coding RNA between the exosomes from treated and nontreated cells showed unique clusters in exosomes from treated vs. non-treated cells. Differential expression of coding and non-coding RNA showed vast changes of expression. As examples, we could identify 6–7 fold upregulation of CTTN and LAMA5, as well as 11 and 6 fold downregulation of PQBP1 and KANK1 respectively.
Conclusion: The inhibition of mutant BRAF induces differential expression of coding and non-coding RNAs in melanoma cells and their released exosomes. This work provides the framework for further investigations of significantly expressed coding and non-coding RNA in exosomes, as well as in cells receiving this cargo.

PF10.08

Antagonistic GTPase signalling regulates the shedding of invasive tumour microvesicles

James Clancy, Christopher Tricarico and Crislyn D'Souza-Schorey

Department of Biological Sciences, University of Notre Dame, IN, USA

Tumour cells utilise a complex and multifaceted approach to degrade and invade through surrounding extracellular matrix (ECM). During invasion through compliant matrices, we have previously demonstrated that invading tumour cells convert to an amoeboid-like mode of invasion, which is accompanied by the extensive release of protease-loaded invasive tumour microvesicles (TMVs) directly into the extracellular

environment. This process is in part facilitated by the activation of the small Ras-related GTPase, ARF6, which regulates the outward flow of recycling membrane and cargo to facilitate TMV formation at the cell surface. Here we extend these findings and show that coordinated and antagonistic regulation of the ARF6 and Rab35 GTPases, in concert with regulation of the cell's contractile machinery, governs TMV shedding from invasive melanoma cells. These results, particularly in light of ARF6 and Rab35 expression in various tumours, highlight the increasing importance of GTPase signalling in the shedding of TMVs, which underlie the morphological and functional changes during adaptive tumour cell invasion.

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PF10.09

Leukaemic-derived exosomes induce paracrine and autocrine cell proliferation in paediatric acute lymphocytic leukaemia

Shabirul Haque and Sarah Vaiselbuh

The Feinstein Institute for Medical Research at Northwell Health, NY, USA

Introduction: Exosomes represent the fingerprint of the parental tumour and are loaded with bioactive markers such as proteins, RNA and DNA, which may regulate tumour growth. Exosomal cargo can be transferred into target cells changing their biological properties. Our data present the first study to investigate a biological role of exosomes in paediatric acute lymphoid leukaemia (P-ALL).

Methods: The aims were to (1) demonstrate that ALL exosomes induce ALL cell proliferation; (2) confirm that exosome induction is regulated by expression of proliferative and pro-survival genes with suppression of pro-apoptotic genes in ALL cells; (3) analyse differences in miRNA profiling of ALL cell lines after induction with leukaemic exosomes

Exosomes were isolated by ultracentrifugation from healthy donors (HD), P-ALL serum and conditioned medium (CM) of SUP-B15 (B-ALL), JM1 (B-ALL), and CL-01 (normal B cells) human cell lines. All cell lines were exposed to different sources of leukaemia-derived exosomes in a paracrine or autocrine fashion. Proliferation was assessed by microscopic cell counting and confirmed by colorimetric assay and gene expression for proliferation (Ki-67, PCNA), pro-survival (MCL1, BCL2) and pro-apoptotic (BAD, BAX) genes. Fold change was calculated by comparing controls (naive) vs. exosome-induced cell lines. miRNA profiling was performed with the Human Cancer Pathway Finder microarray (Qiagen).

Results: We elucidated that CM-derived exosomes from ALL cell lines as well as from serum of ALL patients induce cell proliferation in SUP-B15, JM1 and CL-01 cells. At a molecular level, we found that exosomes from JM1 and SUP-B15 cells enhance expression of proliferation genes (PCNA, Ki-67) and pro-survival genes (MCL-1, BCL2), and suppress pro-apoptotic genes (BAD, BAX) in JM1 cells. Heatmap analysis of miRNA profiles of ALL cell line exosomes will be discussed.

Conclusion: Our data suggest that ALL exosomes induce cell proliferation in both paracrine and autocrine fashion of leukaemic and non-leukaemic B cell lines. Exosomes regulate these phenomena in a highly orchestrated way, by induction of proliferative and pro-survival genes, with suppression of pro-apoptotic genes.

Poster Session F11 – Cell and Tissue Remodelling and Repair
Chairs: TBD 5:15–6:30 p.m.

PF11.01

Human embryonic stem cell-released extracellular vesicles: effects on cultured retinal Müller glial cells and NMDA-damaged mouse retinas, *in vivo*

Edouard Baulier, Yingqian Peng, Yifeng Ke and Debora B. Farber

University of California, Los Angeles, CA, USA

Introduction: We have previously demonstrated that cultured retinal progenitor Müller cells exposed to mouse embryonic stem cell (ESC)-derived extracellular vesicles (ESEVs) show gene expression changes associated with de-differentiation and activation of a retinogenic programme of differentiation. Now we investigated whether human ESEVs (hESEVs) and their fractionated microvesicles (MVs) and exosomes (EXOs) cause similar effects on cultured human Müller cells and rescue *in vivo* the function of damaged mouse retinas.
Methods: hESEVs, MVs and EXOs from culture medium of H9 hESCs were characterised (size, molecular and biochemical components and surface proteins). Their uptake by human cultured Müller cells and their effects on the biochemical components of these cells were studied using imaging flow cytometry, and qRT-PCR, western blots and immunocytochemistry, respectively. Mice with both retinas NMDA-damaged were injected in left eyes with hESEVs and in right eyes with PBS (control). Electroretinograms (ERGs) were measured in each retina 10, 30 and 60 days post-injection.
Results: MVs and EXOs differed in size, RNA profiles, multiple expressed genes and surface markers. hESEVs, MVs and EXOs were all internalised by cultured Müller cells, but only hESEVs and MVs induced changes in the cells (increase of pluripotency mRNAs and proteins) leading to de-differentiation (reflected in a decreased level of Müller cell marker vimentin) and increased level of early retinal protein PAX6 (possibly revealing trans-differentiation of Müller cells into retinal neurons). 2 out of 5 mice that had lost retinal ganglion and amacrine cells after NMDA damage showed great improvement in the ERGs' b-wave amplitude 30 and 60 days after an hESEV injection (which indicated recovery of retinal function). No effect was observed in the PBS-injected retinas.
Conclusion: Exposure to hESEVs or MVs induces molecular changes in human cultured Müller cells leading to their de-differentiation and trans-differentiation into retinal neurons. In initial studies, hESEVs injected into NMDA-damaged retinas of 5 mice, possibly acting through the endogenous Müller cells, rescued retinal function in 2 animals. These are promising findings for future therapy of retinal degenerations.

PF11.02

Hyperglycemia induced microvesicles control endothelial cell migration

Anna Elżbieta. Drożdż[1], Robert Jach[2], Hubert Huras[3] and Ewa Stępień[1]

[1]Department of Medical Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University, Krakow, Poland; [2]Department of Gynaecological Endocrinology, Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland; [3]Department of Obstetrics and Perinatology, Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland

Introduction: Cell migration is one of the crucial mechanisms for wound healing and tissue regeneration. It strongly depends on the biochemical composition of environment and cell vitality. During diabetic diseases cell migration is impaired, which could be linked to the influence of microvesicles (MVs), which are involved in this process.
Methods: Studies were performed using human umbilical vein endothelial cells (HUVECs). The scratch assay was used to assess cell migration in different conditions. A confluence index (CI) was chosen as a parameter to assess cell migration. Culture media containing additional factors: glucose (25 mM/ml or 50 mM/ml) and MVs isolated from plasma of (a) uncontrolled diabetic patients (UD) or (b) healthy control (HC), as well as from the (c) hyperglycemic (25 mM/ml) and (d) normoglycemic HUVEC preconditioned media. Scratch assay was performed, HUVECs were cultured in the density of 42×10^3 cells/cm^2 and recorded immediately and at several time points in the next 14 h. As a long time assessment to confirm dynamics in cell metabolism and proliferation, viability tests were performed. MV concentration in culture medium was flow cytometry tested in the range of 2–4 mln/mL. This study has permission of the Bioethical Committee of Jagiellonian University (KBET/206/B/2013 and 122.6120.78.2016)
Results: Preliminary results showed that in normoglycemic conditions cell migration is higher in presence of MVs from HC compared to the control without MVs (CI: 94.33 ± 6% vs. 81.52 ± 9.47%, respectively). In hyperglycemic conditions cell migration was dysregulated, CI: 53.34 ± 12.85% in presence of UD MVs vs. 81.52 ± 12.8% in the control medium. No differences in cell metabolism and proliferations were observed in the viability tests.
Summary: Endothelial cell migration appears to be controlled by MVs. If MV were isolated from hyperglycemic conditions efficiency of migration was lowered which could be the reason of impaired wound healing process in patient suffer from diabetics disease.

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PF11.03

Withdrawn at author's request.

PF11.04

Novel cell wall remodelling functions of extracellular vesicles secreted by *Saccharomyces cerevisiae*

Kening Zhao[1], Mark Bleackley[1], David Chisanga[2], Michael Liem[1], Hina Kalra[1], Shivakumar Keerthikumar[1], Ching-Seng Ang[3], Christopher Adda[1], Lahiru Gangoda[1], Lanzhou Jiang[1], Ivan Poon[1], Peter Lock[1], Marilyn Anderson[1] and Suresh Mathivanan[1]

[1]La Trobe Institute for Molecular Science, Melbourne, Australia; [2]La Trobe University; [3]Bio21 Institute, University of Melbourne, Melbourne, Australia

Introduction: Extracellular vesicles (EVs) are membranous vesicles released into the extracellular environment by cells. EVs contain various functional cargo that play crucial roles in intercellular communications. In mammalian studies, it is well established that the endosomal sorting complex required for transport (ESCRT) machinery is responsible for the biogenesis of exosomes, an important subtype of EVs with endocytic origin. The ESCRT machinery was first described for its involvement in endosome formation using yeast as a model. Mechanisms of yeast EV biogenesis and release remain unclear, thus further investigations on the characterisation of yeast EVs are required.
Methods: EVs were isolated by differential centrifugations from model yeast *Saccharomyces cerevisiae* upon knockout of selected ESCRT components. Protein quantification, electron microscopy, nanoparticle tracking analysis and label-free quantitative proteomics analysis were done to characterise these EVs. EVs from yeast cultures upon specific drug treatments or gene deletions were also collected for proteomic analysis. Confocal microscopy and flow cytometry were done to investigate EV uptake in yeast. Survival assays with functional EV uptake were then carried out for wild type and cell wall mutant yeast strains upon drug-induced cell wall stress.
Results: It was revealed that EVs isolated from ESCRT knockout cells were altered in terms of protein amounts, protein cargo, morphologies as well as size compared to wild type cells. These differences are especially prominent in EVs upon knockouts of three Vps proteins.

Proteomics analysis promoted further characterisations of different types of yeast EVs and highlighted the enrichment of functional components related to cell wall remodelling in EVs. The presence of EV uptake in yeast was observed, and functional uptake analysis confirmed cell wall remodelling properties of yeast EVs.
Conclusion: This study confirms that yeast EVs can mediate cell wall remodelling under stressful conditions.

PF11.05

Mesenchymal stem cells from chronic kidney disease patients produce extracellular vesicles with increased angiogenic potential

Bas W.M. van Balkom, Femke C.C. van Rhijn Brouwer, Hendrik Gremmels, Vidalmar Briceno and Marianne C. Verhaar

UMC Utrecht

Introduction: Mesenchymal stromal cell (MSC) therapy is used for a variety of degenerative and immunological diseases. A fundamental question is whether co-existing disease affects the regenerative properties of autologous cells. MSCs exert their regenerative properties through paracrine secretions, with a major role for extracellular vesicles (EV). We investigated whether chronic kidney disease (CKD) affects the angiogenic potential of MSC-derived paracrine factors.
Methods: Bone marrow from patients scheduled for living donor kidney transplant (CKD) and from persons donating a kidney (healthy controls) was obtained for subsequent MSC isolation and culturing. The study was approved by the local medical ethical committee and all MSC donors provided written consent. We determined angiogenic potential of conditioned medium and isolated EVs by *in vitro* matrigel angiogenesis analysis. EVs were isolated by sequential centrifugation and presence and purity were assessed by nanoparticle tracking analysis, sucrose density gradient centrifugation and immunoblotting.
Results: MSCs from three controls and three CKD patients were cultured up to passage 8 and conditioned medium was collected for angiogenesis assays and EV isolation. Isolated EVs had a density of 1.1 g/mL, a nominal size of 144 nm and contained the typical EV marker Flotillin-1, and nuclear and mitochondrial proteins were absent, indicating their purity. MSC-conditioned medium from both controls and patients stimulated angiogenesis. No differences could be observed between the two. Interestingly, isolated EV from CKD patient MSCs potently stimulated angiogenesis, whereas no vessel formation could be observed after stimulation with EV from control MSCs.
Conclusion: EV from patient MSCs show a higher angiogenic potential than those from healthy control MSCs. This effect of disease state on MSC-derived EV function could be attributed to differences in EV secretion or EV content.

PF11.06

Exosomes secreted by human adipose-derived stem cells regulate the expression of collagen synthesis-related genes in human dermal fibroblasts

Yeo Jin Choi, Kyoung Soo Lee, Seung Ho Yeom and Yong Woo Cho

Hanyang University, Seoul, Republic of Korea

Introduction: Human skin ageing is a complex biological process influenced by intrinsic (e.g., hormone and metabolic processes) or extrinsic factors (e.g., ultraviolet radiation). In particular, UV-induced ageing causes significant downregulation of collagen synthesis and induction of matrix metalloproteinases (MMPs) expression in skin, leading to wrinkle formation. Human adipose-derived stem cells (HASCs) secrete exosomes containing nucleic acids (mRNAs and microRNAs) and bioactive proteins, which could act as critical signals of tissue regeneration. Herein, we hypothesised that HASCs-derived exosomes may have a positive role in promotion of collagen remodelling in UVB-induced skin damage.
Methods: Exosomes were isolated from conditioned media during HASC proliferation through pre-filtration in 0.2 μm, followed by tangential flow filtration (TFF) system (500 kD MWCO). The isolated exosomes are characterised by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and western blot analysis. Exosomal miRNA were profiled using miRNA arrays containing probes for 2578 human mature miRNAs and cytokines were analysed using human 80 cytokine array kit. The potency of exosomes was evaluated by a monitoring of the cellular behaviours and expression of collagen synthesis-associated genes in UVB-exposed dermal fibroblasts.
Results: The exosomes were approximately 50–120 nm in diameter and expressed exosomal markers such as CD9 and CD81. Exosomal miRNAs and various cytokines related to skin reconstruction were identified in exosomes. We found that exosomes significantly promoted fibroblast migration in a scratch assay. Interestingly, exosome treatment reduced UVB-induced *MMP-1* gene expression and increased gene expression of tissue inhibitor of megalloproteinase-1/-3 (*TIMP-1/-3*) and collagen type I alpha 1 (*COL1A1*).
Conclusion: Our findings suggest that HASC-derived exosomes act as a biological cue stimulating dermal fibroblasts and might be used as a potential agent for skin rejuvenation.

PF11.07

Co-delivery of multiple miRNA cargos to enhance therapeutic vascularisation bioactivity of extracellular vesicles

Anjana Jeyaram and Steven M. Jay

University of Maryland, College Park, MA, USA

Introduction: Extracellular vesicles (EVs) are promising candidates for therapeutic miRNA delivery, however challenges related to large scale production of desired vesicles as well as relatively low and variable levels of endogenous miRNAs pose obstacles for eventual clinical translation. Thus, it is desirable to controllably engineer EV content via extrinsic loading methods. As multiple pathways regulate disease conditions, we believe that actively loading multiple cargos can effectively enhance the therapeutic bioactivity of EVs.
Methods: Human embryonic kidney cell (HEK293T)-derived EVs were used to deliver miRNAs since they are believed to be relatively inert and do not enrich disease-related pathways. Known inducers of vascularisation, miR-93 and miR-126, were selected as cargo molecules. EVs were loaded via sonication, as we previously demonstrated its advantages over electroporation. After using fluorescent measurements to quantify miRNA loading after sonication, human umbilical vein endothelial cells (HUVECs) were used to assess vascularisation potential of EVs in a wound healing migration assay. Western blots of cell lysates demonstrate signalling regulation.
Results: A dose-dependent increase in HUVEC migration area was observed for cells treated with each of the miRNA loaded EVs. While cells treated with only miR-93 loaded EVs or only miR-126 loaded EVs showed 50% and 69% closure of the wound area, respectively, cells treated with EVs with a combination of two miRNAs induced 81% wound area closure. Treatment of cells with HEK293T-derived EVs without miRNA cargo showed only 39% wound area closure.
Conclusion: Delivery of pro-vascular miRNAs via EVs can potentially enable the revascularisation of chronic wounds and ischemic tissues. Overall, this study points to potential benefits of co-delivery of miRNA cargos targeting different gene regulation pathways towards vascularisation. Ongoing work will assess whether loading multiple miRNAs within the same EV is more effective than using multiple populations of EVs loaded with different cargos.

PF11.08

NOS1AP coded protein, capon, is required for leucocyte microparticle production and inflammasome activation in response to hyperglycemia

Stephen R. Thom[1], Veena Bhopale[1] and David Margolis[2]

[1]University of Maryland School of Medicine, MA, USA; [2]University of Pennsylvania School of Medicine, PA, USA

Introduction: Up to 20% of diabetic patients develop a foot ulcer (DFU) and most who have a lower extremity amputation (LEA) will have had a

DFU. We demonstrated in diabetics that Nitric oxide synthase 1 adaptor protein (*NOS1AP)* gene variation is associated with LEA. Function of the *NOS1AP* coded protein, capon, is unknown outside the nervous system. We hypothesised that hyperglycemia stimulates leukocytes to produce microparticles (MPs, 0.1–1 μm diameter, annexin V-positive) and activates the nucleotide-binding domain-like receptor 3 (NLRP3) inflammasome due to oxidative stress, and capon has a role.
Methods: Human and murine leukocytes were incubated *ex vivo* in buffer containing 5.5–20 mM glucose, the buffer and cells separated for flow cytometer MPs analysis and biochemical assays. Capon content was manipulated using small inhibitory RNA.
Results: Hyperglycemia (>11 mM) increased neutrophil mitochondrial reactive oxygen species production and activity of NADPH oxidase. Concomitant activation of type-2 nitric oxide synthase (NOS) occurs with secondary oxidants resulting in actin S-nitrosylation and enhanced filamentous actin turnover, followed by increased MPs production. Oligomerisation of inflammasome components including Apoptosis-associated Speck protein with CARD domain, NLRP3 and caspase 1 occurs leading to IL-1β synthesis and packaging within MPs. Immunoprecipitation shows capon is required for NOS linkage to short filamentous actin. Capon depletion prevents hyperglycemia-induced NOS activation, actin turnover, MPs formation and NLRP3 activation.
Conclusion: Capon links NOS to the cytoskeleton. It is required for enhanced reactive species formation and consequent production of MPs containing IL-1β. MPs are elevated in diabetes and hyperglycemia can activate the NLRP3 inflammasome, which contributes to development of diabetic vasculopathy. We hypothesise that gene variations modify capon causing a gain of NOS function that exacerbates risk for LEA.

PF11.09

Transfer of extracellular vesicles between fibroblasts and keratinocytes in cellular senescence

Madhusudhan Reddy Bobbili[1], Lucia Terlecki Zaniewicz[2], Markus Schosserer[1], Vera Pils[2], Dietmar Pum[3] and Johannes Grillari[2]

[1]University of Natural Resources and Life Sciences, Department of Biotechnology; [2]Christian Doppler Laboratory for Biotechnology of Skin Ageing, Department of Biotechnology, BOKU University Vienna, Austria; [3]University of Natural Resources and Life Sciences, Institute of Nanobiotechnology

Introduction: Extracellular vesicles emerged as an important mode of cell-to-cell communication in both normal and pathological conditions. Extracellular vesicles regulate the target cell by releasing their cargo – RNA, proteins and metabolites, which they carry from the cell they originate.
Methods: We observed that extravesicular (EV)-miRNAs secreted from fibroblasts are taken up by keratinocytes. Our main aim, is to identify senescence-associated extravesicular (SA-EV) small RNAs, especially EV-miRNAs and their impact on keratinocyte functionality. To achieve this, stress-induced premature senescence (SIPS) was triggered in human dermal fibroblasts (HDF) and EVs below 220 nm in diameter were harvested by differential centrifugation.
Results and Conclusion: Thereby, we have observed that senescent HDFs secrete more than 4-fold more exosome like vesicles per cell, and that these EVs show hallmarks of exosome. In addition, to an EV-transfer in 2D, we also observed a miRNA crosstalk in an *in vivo* mimicking 3D cell culture model. We further analysed the EV-miRNA signature and identified highly secreted candidates that might be involved in keratinocyte differentiation and wound healing.

LBP.27

Placental trophoblast debris mediated feto-maternal signaling via small RNA delivery: implications for preeclampsia

Jia Wei[1], Cherie Blenkiron[2], Peter Tsai[3], Joanna James[3], Qi Chen[3], Peter Stone[3] and Lawrence Chamley[3]

[1]The University of Auckland, New Zealand; [2]Department of Molecular Medicine and Pathology, University of Auckland, Auckland, New Zealand and School of Biological Sciences, University of Auckland, Auckland, New Zealand; [3]University of Auckland, New Zealand

Introduction: During pregnancy the outer layer of the placenta, the trophoblast, sheds large quantities of debris into the maternal circulation. These macrovesicles (MaV) have an important signaling role in maternal cardiovascular adaptation to pregnancy in part through modulation of recipient endothelial cells. We hypothesized that the small RNA cargo of MaV would be involved in this signaling, a process which may be modified in the hypertensive disease preeclampsia.
Methods: Placenta were collected from term normotensive (n=13) or preeclamptic (n=10) pregnancies with written consent from the donors under National Ethics committee project approval NTX/12/06/057. MaV were collected from placental explant cultures by centrifugation after transfection with artificial small RNAs and delivery of Cy3-labelled RNAs was visualized by confocal microscopy or validated by qRT-PCR. The small RNA content of placenta MaV (n=5 each group) was determined by small RNA-seq and analysed using the published iSRAP pipeline.
Results: Explant cultures showed uptake of a control Cy3-labelled small RNA into the placental tissue, with efficient packaging into deported MaV and subsequent delivery into MaV treated recipient endothelial cells. Small RNA-seq identified the contents of MaV differed between healthy term and preeclamptic placenta with differential abundance of 16 miRNAs (including miR-145), 5 tRNA fragments, 13 snRNA fragments and 85 rRNA fragments. There was also evidence of selective packaging of selected small RNAs into the MaV from the placenta. Finally, engineered healthy placental MaV were able to deliver synthetic miR-145 into endothelial cells which induced transcriptional changes in endothelial cells similar to those induced by preeclamptic MaV.
Summary/Conclusion: Macrovesicles deported into the maternal circulation might deliver small RNA to maternal cells and contribute to feto-maternal communication. These small RNAs are dysregulated in preeclamptic MaV and may contribute to the endothelial cell activation, a hallmark of preeclampsia.
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LBP.28

Placenta-specific microRNAs in circulating exosomes showed different levels in pregnancies complicated by preeclampsia

Virginie Gillet, Larissa Takser and Annie Ouellet

Université de Sherbrooke, Sherbrooke, Montreal, Canada

Introduction: Preeclampsia (PE) is a pregnancy-specific syndrome and one of the leading causes of maternal and fetal morbidity and mortality. Even if the pathophysiological mechanisms remain poorly known, placental dysfunction is involved in pathogenesis and clinical signs (hypertension and proteinuria) appear during 2nd or 3rd trimester. Several studies revealed differentially expressed microRNAs with either high or low levels of expression in human placentas from normal versus preeclamptic pregnancies. Recent studies reported that placental-specific miRNAs belonging to C19 miRNA cluster (C19MC) were released into maternal circulation through exosomes and could represent new avenue for biomarker discovery.
Methods: Methods: We performed a case-control study in a cohort of 40 pregnant women enrolled during their third trimester of pregnancy, in Centre Hospitalier Universitaire, Sherbrooke, Canada. Plasma samples from 10 women with PE, 10 high-risk women for PE but who did not develop the disease and 20 women with a normal pregnancy were analysed. The concentration of placental exosomes was quantified using a commercial ELISA kit. Circulating exosomes (including placental exosomes) and microRNAs were purified from maternal blood using ExoRNeasy method and relative expression of the C19MC microRNAs was done by qRT-PCR home-made assays.
Results: Results: C19MC placenta specific miRNAs (mir-515-5p, miR-517-5p, miR-517a, miR-518b, miR-520a, miR-520h and miR-525-5) were detected in our samples confirming the presence placental-EVs in maternal blood. Despite a slightly increase level of placental EVs for PE cases compared to normal pregnancies and high-risk pregnancy, results

did not reach significance. In PE pregnancies we report over-expression of miR-525-5p, miR-517a and miR-520a (fold change = 1.3; 1.4 and 7 respectively) compared to both high-risk and normal pregnancies. On the contrary, miR-515, miR-517-5p, miR-518 and miR-520h levels are diminished by 2-fold compared to both high-risk and normal pregnancies. No difference was observed between miRNAs levels between high-risk and normal pregnancies, tending to support a specific miRNA signature for PE. Summary/Conclusion: Conclusion: Exosomes as well as miRNAs could represent a new avenue in the area of diagnostic of pregnancy complications related to placental dysfunction.

LBP.29

Evaluation of a profile of exosomes and MiRs playing roles in the pathogenesis of human corneal endothelial dysfunctions

Junji Hamuro[1], Kazuko Asada[2], Morio Ueno[2], Chie Sotozono[2], Takahiro Ochiya[3] and Shigeru Kinoshita[4]

[1]Department of Ophthalmology; [2]Dept Ophthalmology; [3]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Japan; [4]Department of Frontier Medical Science and Technology for Ophthalmology

Introduction: Cultured human corneal endothelial cells (cHCECs) serve as an alternative to donor corneas for the medication of corneal endothelial dysfunction. However, predisposition of cHCECs into a senescence phenotype, epithelial-mesenchymal transition (EMT) have been hampering the practice in clinical settings.
Methods: The variations of cHCECs in their composites of heterogeneous SPs were certified in the context of their surface CD markers. The integrated analysis of miRNA profiles contained in secreted exosomes were investigated by 3D-gene (Toray). The exosomes were analyzed either by western blotting, exoscreen or FACS for CD9 CD63 CD81 and EpCAM. The function of detected miRs was validated by transducing them into heterogeneous SPs of cHCECs.
Results: Secreted exosome profiles among morphologically diverse CHCEC SPs proved to be useful for their distinction each other. The CD44 negative SP suitable for injection therapy expressed only CD63, while the CD44 intermediate Sp expressed CD81 in an inverse correlation with the extent of CD44. The CD44 strongly expressing cHCEC Sp with cell state transition (CST) secreted CD9CD63 double positive exosome. The candidate miRs included in exosomes, able to discriminate CD44- SPs from those with CD44+++ phenotypes were 1246 and 1273e (inverse relation with CD44 expression), and 24-3p and 184 (positive correlation with CD44). Of note, intracellular miR184 only decreased inversely in parallel with the upregulation of CD44 on cHCECs. CD9 CD63 double positive exosomes secreted far abundantly by cHCEC Sps with CST were far more incorporated into both of cHCECs with or without senescence or EMT-like CST, indicating the presence of new pathway of synchronized cell state conversion into pathogenic phenotypes, by intracellular export of extracellular vesicles (EVs) into cHCECs without CST.
Summary/Conclusion: The cHCECs sharing a CD44- phenotype may be discriminated by the profile of exosomes secreted. Thus miRs in secreted exosomes may serve as the tool to qualify cultured cHCECs. The precise analysis of the proposed cell to cell communication through EVs might open the new aspect for the better understanding of pathogenesis of bullous keratoplasty.
Funding: This research is supported by the Highway Program for Realization of Regenerative Medicine from AMED, JSPS KAKENHI JP26293376 and Core to Core programme, AMED, Japan

LBP.30

Role of tumor-derived exosomes in immunosuppression in malignant melanoma

Viktor Fleming

German Cancer Research Center, Heidelberg, Germany

Introduction: Malignant melanoma is the most dangerous form of skin cancer and accounts for almost 80% of all skin cancer deaths. The accumulation of highly immunosuppressive myeloid-derived suppressor cells (MDSCs), which arise from immature myeloid cells (IMC) in the bone marrow, play a significant role in immunosuppression and in the resistance to immunotherapy of malignant melanoma. It was shown that melanoma cells can recruit MDSC by secreting exosomes.
Methods: TEX were isolated *in vitro* from RET-murine melanoma cell line by serial centrifugation steps and characterized via western blot for exosomal markers. In addition, we performed nanoparticle tracking analysis for the size distribution of the isolated vesicles. To investigate the effects of TEX on IMC, IMC were isolated from the bone marrow of wildtype C57BL/6 mice via magnetic sorting. Those cells were either prepared for flow cytometry, Western blot, ELISA or qPCR analysis.
Results: We have previously shown that injection of TEX derived from the murine RET melanoma cell line induced the accumulation of IMC in the bone marrow after injecting TEX into wildtype C57BL/6 mice. TEX induced the activation of STAT3 and NFkB in IMC. Moreover, the treatment with TEX was sufficient to block the differentiation of IMC into mature myeloid cells. Instead, the treated IMC were producing inflammatory cytokines such as IL-1β, IL-6, IL-10, TNF-α and VEGF. In addition, a strong upregulation of PD-L1 was measured. By studying myD88 knock-out mice, we found that these alterations were mediated by the stimulation of the NFkB signaling pathway. TEX-treated IMC could also inhibit the proliferation of $CD8^+$ T cells and reduce the production of interferon-γ. Interestingly, the impact of TEX-treated IMC on T cell functions was not mediated by the NFkB pathway.
Summary/Conclusion: Taken together the results confirm that TEX play an import role in the tumor progression. Melanoma cells use exosomes to dampen the immune system by converting myeloid cells into an immunosuppressive phenotype. Furthermore, increased amounts of TEX leads to an accumulation of immature myeloid cells in the bone marrow. The signaling pathways involved in the TEX-mediated conversion of IMC into MDSC-like cells are now under investigation.

LBP.31

The diagnostic potential of sentinel extracellular vesicles in early inflammation

Revathy Munuswamy[1], Sören Kuypers[1], Jan D'Haen[2], Inge Nelissen[3], Joy I. Irobi[1], Baharak Hosseinkhani[1] and Luc Michiels[1]

[1]Hasselt University, Biomedical research institute, Martelarenlaan 42, 3500 Hasselt, Belgium; [2]IMO-IMOMEC, Hasselt University, Wetenschapspark 1, 3590, Diepenbeek, Belgium; [3]VITO NV, Boeretang 200, 2400 Mol, Belgium

Introduction: Inflammation is involved in the onset of several diseases such as Alzheimer, allergies and cardiovascular disease. Recent evidence reveals a strong association of monomeric C-reactive protein (mCRP) in the early inflammation process and we demonstrated the presence of mCRP on Extracellular Vesicles (EV) produced by inflamed cells. EV play a pivotal role in the process of initiation, propagation and regulation of inflammation. However, the precise role of mCRP in the physiological and pathological functions of EV and their potential as biomarkers in inflammatory conditions is not known yet. Our aim is to address the question whether mCRP carrying EV can serve as a potential sentinel marker for early inflammation.
Methods: Primary endothelial cells (HUVEC) were cultivated either unstimulated or triggered for inflammation using TNF-α. EV were isolated from supernatant of both HUVEC cultures using size exclusion chromatography (SEC). Different tools such as an immunofluorescence (IF) assays, western blot (WB), TEM and NTA analysis were performed to characterize and to confirm successful isolation of EV from both conditions. mCRP carrying EV were analyzed by binding to a mCRP specific aptamer using label free, surface plasmon resonance (SPR).
Results: Vesicles having an approximate size range between 100-200 nm were successfully isolated using SEC. SPR analysis showed a fivefold increase of mCRP+ EV in TNF-α treated HUVEC cultures as compared to untreated cells. The observed changes were confirmed using WB, TEM and IF techniques. Moreover the WB analysis also showed the presence of EV classical markers such as CD9. Using fluorescent labeled aptamer we demonstrated the ability of inflamed EV to transport mCRP to untreated HUVEC cells triggering this way a pro-inflammatory status in the recipient cell.
Summary/Conclusion: Our current study confirms that the circulating EV have a great potential as a sentinel tool in early inflammation. This

study also opens up the opportunity to develop a reliable, reproducible and robust tool to detect circulating mCRP EV in diagnostic application.
Funding: This work was financed by Hasselt University and by EFRO through the Interreg V Grensregio Vlaanderen-Nederland project Trans Tech Diagnostics and the Marie-Curie Initial Network programme, r'BIRTH project (grant agreement no. 608346) from the EU.

LBP.32

Extracellular vesicles derived from monocytic THP-1 and SW480 colon cancer cells induce pro-inflammatory response in human primary monocytes

Tonje Bjørnetrø[1], Kari Bente Foss Haug[2], Beate Vestad[2], Lilly Alice Skaaraas[2], Anne-Marie Trøseid[2], Hans Christian D Aass[2], Alicia Llorente[3] and Reidun Øvstebø[2]

[1]Institute of Clinical Medicine, University of Oslo, Norway; [2]The Blood Cell Research Group, Department of Medical Biochemistry, Oslo University Hospital, Ullevål, Norway; [3]Oslo University Hospital-The Norwegian Radium Hospital, Oslo, Norway

Introduction: Extracellular vesicles (EV) represent an important mode of intercellular communication by serving as vehicles for molecular transfer between cells. The specific functions of EV on target cells depend on the ability of EV to interact with recipient cells, delivery of their specific contents and initiating downstream signaling. The present study has investigated if THP1- and SW480-derived microvesicles (MV) and exosomes (EXO) are able to enter and activate an inflammatory response in human primary monocytes.
Methods: Collection and isolation of EV: THP-1 (human leukemia monocytic) and the SW480 (human colon adenocarcinoma) cells were cultured at 37°C, 5% CO_2 in serum-free RPMI media for 24 hours. Subpopulations of EV were obtained from sequential centrifugation of the 4500xg supernatant; in particular MV were pelleted by 17000xg, 30 min and EXO obtained by filtration of the 17000xg supernatant with a 0.22mm filter (Millex GV) and concentrated by a 100kDa Centricon filter (Amicon ®Ultra-4). Particle size and concentration of EV were analyzed by NTA.
Functionality of EV in human primary monocytes: Elutriation-purified, cryopreserved monocytes (1.5 x 10^5 in150 mL) from healthy donors were thawed and re-suspended in 10 % (v/v) FCS-RPMI. MV and EXO (10^{10}-10^8) (derived from THP-1 and SW480 cells) ± fluorescently labeled with PKH67 (Sigma Aldrich) were incubated with monocytes for 4 hours at 37°C, 5% CO_2. Subsequently, the supernatants were harvested and stored at -80°C until the secretion of IL1-b, IL6, IL8, TNF-a, MCP-1, MIP-1b and IP10 proteins (Luminex) were analyzed. The uptake of EV in monocytes was analyzed by flow cytometry (BD Accuri C6) and fluorescence microscopy/live imaging (Nikon Eclipse Ti).
Results: THP-1 and SW480 derived MV and EXO were all internalized by human primary monocytes in a dose-dependent manner. The exposure of EV induced a dose-dependent secretion of IL1-b, IL6, IL8, TNF-a, MCP-1, MIP-1b and IP10 from the monocytes. Our data show that MV and EXO derived from different cell lines affect the secretion of inflammatory molecules to different extents.
Summary/Conclusion: Extracellular vesicles derived from THP-1 and SW480 cells are internalized and induce inflammatory responses in human primary monocytes.
Funding: Regional Research Network on Extracellular Vesicles, South-Eastern Norway Regional Health Authority

LBP.33

Exosomal miRNA in Hep2G cells stimulated by pro-inflammatory cytokines

Justyna Totoń-Żurańska[1], Michał Seweryn[1], Katarzyna Poskrubek[2], Anna Wiśniewska[2], Rafał Olszanecki[2], Pawel Wołkow[3] and Ryszard Korbut[4]

[1]Jagiellonian University Medical College Center for Medical Genomics - OMICRON, Krakow, Poland; [2]Jagiellonian University Medical College Department of Pharmacology, Krakow, Poland; [3]Jagiellonian University Medical College Center for Medical Genomics - OMICRON, Jagiellonian university Medical College Department Of Pharmacology, Krakow, Poland; [4]Department of Pharmacology Jagiellonian University Medical College, Krakow, Poland

Introduction: Mutual interplay between Kupffer cells (KCs) and hepatocytes plays a role in the development of non-alcoholic liver steatosis and steatohepatitis. Excessively activated by lipid accumulation Kupffer cells (KCs) release a large amount of pro-inflammatory cytokines, which are harmful to hepatic cells. Other way around, hepatocytes secrete multiple factors with potential influence on KCs. The aim of our study was to assess exosomal miRNA cargo of hepatic cells primed *in vitro* by inflammatory stimuli in order to identify miRNA, which potentially could in response regulate expression of transcripts involved in KCs. Additionally, in this setting we assessed the action of sylimarin – the compound with recognized mild hepatoprotective action.
Methods: We have performed sequencing of exosomal miRNA from Hep2G cells treated with: TNF-alpha, INF-gamma, silimarin. We have used EdgeR' to detect transcripts differentially regulated between conditions. We aimed to detect non-linear effects of treatment with silimarin. We have detected octamer RNA sequences which are over-represented in the exosomes from stimulated cells and we have used these motifs to detect mRNAs related to lipid metabolism which are more likely to be the targets of micro RNAs with these specific motifs. We validated our predictions using the available databases for miRNA-target interaction prediction (e.g. PITA, TargetScanS). We have used Renyi Divergence of order 0.5 to quantify the similarity of expression patterns of all transcripts, to a pre-selected set of miRNAs known to be involved in inflammatory pathway, across all experimental conditions. We have performed hierarchical clustering to detect co-regulated micro RNAs.
Results: Among most significantly changed by inflammatory stimuli exosomal miRNAs were: hsa-let-7b-5p, targeting IRS2, involved in steatohepatitis, hsa-miR-6790-5p and hsa-miR-146b-5p, whereas silimarin affected exosomal presence of hsa-miR-146b-5p, hsa-miR-542-3p. mir-146b was reported to directly influence TRAF6 and IRAK1 mRNA and protein levels in macrophages
Summary/Conclusion: We have identified set of miRNAs, which presence in Hep2G exosomes is altered by inflammatory stimuli and which could potentially affect expression of genes involved in lipid metabolism in KCs. The exact influence of Hep2G-derived miRNA on KCs require further investigation.

LBP.34

Platelet derived-microparticles as modulator of plasmacytoid dendritic cell inflammatory response

Adam Ceroi[1], Sameh Obeid[2], Thomas Cherrier[3], Céline Elie-Caille[2], Wilfried Boireau[2] and Philippe Saas[3]

[1]Ravicahndran's Lab., University of Virgina, VA, USA; [2]Institut FEMTO-ST, CNRS, Univ. Bourgogne Franche Comte; [3]INSERM UMR 1098, ESF-Bourgogne-Franche Comté, Univ. de Franche-Comte

Introduction: Microparticles (MP) are generated from the plasma membrane of parental cells after activation or cell death. Depending on the stimulus responsible for MP generation, it was demonstrated that the quantity, content and biological activities of MP may vary. Previously, we demonstrated that platelet or endothelial cell-derived MP uptake by plasmacytoid dendritic cells (PDC) can modulate the Liver X Receptor (LXR) pathway, and then regulate inflammatory responses. Here, we used MP from resting- or collagen activated-platelet (rest-PMP or col-PMP, respectively) to compare their size and protein content, and investigate their effect on the LXR pathway and the subsequent inflammatory response in PDC.
Methods: Platelet from healthy donors were stimulated or not by collagen, and platelet derived-MP (PMP) were isolated by centrifugation. PMP size and concentration were investigated by flow cytometry and Surface Plasmon Resonance coupled with Atomic Force Microscopy, followed by Mass Spectrometry to ask their protein content. Using PDC from healthy donors, we investigated LXR pathway involvement (through LXR-target gene expression: *LXRA, SREBF1* and *ABCA1*) and inflammatory cytokine transcription (*TNFA* and *IL8*) in these cells after culture with PMP.
Results: Rest-PMP showed a significant bigger size associated with a specific protein content. Culture of PDC with rest-PMP activated

significantly the LXR pathway and repressed *IL-8* and *TNFA* transcription. Interestingly, culture of PDC with col-PMP induced the opposite effect, with a significant repression of the LXR pathway and an increase of inflammatory cytokine gene transcription.
Summary/Conclusion: These data demonstrated that collagen stimulation of platelets induced PMP with modified size and protein content, able to induce a PDC inflammatory response in culture. Since collagen-stimulated platelets and PDC are involved in rheumatoid arthritis, the col-PMP-induced PDC inflammatory response might be a new therapeutic approach in this context.
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LBP.35

Microvesicles, an anti-inflammatory player, compromised in asthmatic patients

Adam Ceroi[1], Alexandra Bettina[1], John Steinke[2], Luca Musante[3], Uta Erdbruegger[4], Larry Borish[5] and Kodi Ravichandran[6]

[1]Ravicahndran's Lab., University of Virgina, VA, USA; [2]Department of Medicine, Allergy and Immunology, University of Virgina, VA, USA; [3]Department of Medicine, Nephrology Division, University of Virgina, VA, USA; [4]Department of Medicine/Nephrology Division, University of Virgina, VA, USA; [5]Department of Medicine, Allergy and Immunology; [6]Microbiology, Immunology, and Cancer Biology Dpt., University of Virgina, VA, USA

Introduction: Alveolar macrophage (AM)-derived microvesicles (MV) reduce inflammatory responses of alveolar epithelial cells in lung (Bourdonnay et al., JEM, 2015). Soluble factors produced by macrophages, such as IGF-1, which promotes the uptake of microvesicles, dampened airway inflammation in mice (Han et al., nature, 2016). Here, we tested the inflammatory effects of MV isolated from broncho-alveolar lavage fluid (BALF) from asthmatic patients, on the airway epithelial cells.
Methods: MV isolated from BALF from asthmatic patients, and AM-MV from alveolar macrophages cell line (MH-S) cultures, were isolated after cell depletion, filtration (0.8 μm), size exclusion chromatography, and precipitation by differential centrifugation. MV size and concentration were assessed by tunable resistive pulse sensing. Two alveolar epithelial cell lines (BEAS-2B and A549) were used to assess MV uptake and transcriptomic modification within the epithelial cells, in the presence or absence of BALF or microvesicles-depleted BALF (BALF-MV$^-$).
Results: BALF from asthmatic patients induced inflammatory cytokines (CSF2, IL-6 and IL-8) in alveolar epithelial cells. Unexpectedly, total microvesicles from BALF (as well as AM-MV) reduced induction of inflammatory cytokines, while microvesicles-depleted BALF-MV$^-$ did not reduce pro-inflammatory cytokines. In contrast, the BALF-MV$^-$ reversed the effect of microvesicles in dampening inflammatory *CSF2, IL-6 and IL-8* expression. These effects of the BALF-MV$^-$ were correlated with a decrease of microvesicle uptake by epithelial cells.
Summary/Conclusion: These data suggest that MV from BALF from asthmatic patients have the potential to dampen the inflammatory cytokine production by alveolar epithelial cells. Nevertheless, this anti-inflammatory effect is reversed by BALF supernatant (i.e. components other than microvesicles), which may involve an inhibition of MV uptake by airway epithelial cells. The identification of the specific BALF components that are responsible for reversing the inherent anti-inflammatory effect of microvesicles could serve as potential therapeutic targets.

LBP.37

Rapamycin-loaded Exosomes: A strategy to enhance drug-delivery to Insulin-producing beta-cells

Miles Brooke[1], Marta Garcia-Contreras[2] and Camillo Ricordi[3]

[1]Diabetes Research Institute; [2]University of Miami, Diabetes Research Institute, FL, USA; [3]University of Miami, Diabetes Research Institute, FL, USA

Introduction: Exosomes are a type of extracellular vesicle that mediate intercellular communication between cells by transporting molecular information. Exosomes have emerged as relevant therapeutic tools and pharmaceutical drug delivery vehicles. The aim of this study was to investigated the ability of exosomes to act as an effective transporter of an immunosuppressant drug, rapamycin, and evaluate their *in vitro* cytotoxicity to MIN6 cells. Rapamycin (sirolimus) is one of the primary immuno-suppressants for islet transplantation. MIN6 cells display characteristics of pancreatic beta islet cells, such as the secretion of insulin, which makes them important in diabetes research.
Methods: We isolated exosomes from the culture medium of MIN6 cells and Adipose-derived Mesenchymal Stem Cells (MSCs) using Exoquick-TC (SBI). Exosomes were characterized by transmission electron microscopy, nanoparticle tracking analysis and Western blot. Rapamycin was loaded into the MIN6 or MSCs-derived exosomes and confirmed by HPLC, the uptake of exosomes by MIN6 cells was assessed by confocal microscopy. Cell death was evaluated using Annexin V/Propidium Iodide with Flow cytometry and an Alamarblue Viability Assay were conducted to measure the cytotoxicity effectiveness of exosomes as a delivery system for rapamycin.
Results: Our results point to exosomes being an effective delivery system for rapamycin into MIN6 cells. The cytotoxic effect of the rapamycin increased when loaded into exosomes as compared to unloaded delivery. As the concentration of rapamycin loaded into the exosomes increased, the percentage of cells that began signaling for cell death increased. The delivery of rapamycin to the target cells was more efficient in the MIN6 derived exosomes than in those from the MSC cells.
Summary/Conclusion: Exosomes are a viable and effective delivery system for drug delivery into MIN6 cells. The loaded exosomes lead to rapamycin having an increased cytotoxic effect than when introduced to MIN6 cells in an unloaded state. Exosomes can be thought as a potential tool for a specific delivery of functional drugs to improve islet transplantation.
Funding: This work was supported by the Diabetes Research Institute Foundation (DRIF).

LBP.38

Exosomes released by Insulin-secreting cells and human islets under stress conditions reveal an altered microRNA profile: Implications for Monitoring Islet transplantation

Marta Garcia-Contreras[1], Alejandro Tamayo[2], Miles Brooke[2], Carlo Bosi[3], Luciarita Boccuzzi[4], Peter Buchwald[5], Paul Robbins[6] and Camillo Ricordi[7]

[1]University of Miami, Diabetes Research Institute, FL, USA; [2]Diabetes Research Institute; [3]University of Milan, Milan, Italy; [4]Florida International Institute, FL, USA; [5]Diabetes Research Institute; [6]The Scripps Research Institute, Jupiter, Florida, USA; [7]University of Miami, Diabetes Research Institute, FL, USA

Introduction: There is a need for non-invasive biomarkers for early detection of beta cell survival, functional integrity, dysfunction and loss after transplantation and following intervention trials to reverse autoimmunity in Type 1 Diabetes Mellitus(T1D). Exosomes (EXOs) have been shown to provide an enriched protective source of miRNAs for biomarker profiling compared to tissue/cellular and plasma/serum sources. The aim of this study was to evaluate the impact of stress conditions, that human islets are exposed in the transplantation period, on the miRNA profile of insulin-producing β-cells and validate their biomarker potential in the clinical setting.
Methods: MIN6 cells and Human islets were cultured for 48 h under standard, hypoxic (3% O_2), or inflammatory conditions (cytokine cocktail of IL-1β, 50 U/mL; IFN-γ, 1,000 U/mL; and TNF-α, 1,000 U/mL). Plasma samples were collected from T1D patients before and after islet transplantation (consenting and ethics approved). EXOs were isolated from conditioned medium using Exoquick-TC (SBI) and from 500 ml of human plasma were isolated by ultracentrifugation. EXOs were characterized by TEM microscopy, Nanoparticle tracking analysis, flow cytometry and western blot. RNA was isolated (miRVana, Ambion) and exosomal miRNA profiling was performed using a Nanostring 800 miRNA array (Nanostring) on MIN6 and islet-derived EXOs and plasma-derived EXOs content was analyzed by RNAseq (SBI).
Results: Insulin-secreting β-cell derived EXOs express a distinct RNA signature compared to stressed cells. A subset of 2/4 and 14/20 miRNAs were differentially expressed in MIN6 EXOs/human-islet EXOs under inflammatory and hypoxic conditions respectively. Preliminary RNASeq

data analysis revealed that islet-derived EXOs miRNAs were found in transplanted patients in relation to allograft injury and function.
Summary/Conclusion: Together, our findings provide strong evidence that exosomes from insulin-secreting cells under stress-induced conditions modify their cargo. Those changes in the exosomes can be detected in immediate islet post-transplantation period, and could be used as biomarkers for assessment and monitoring in-vivo beta cell functional integrity, dysfunction, and loss.
Funding: This work was supported by the NIH-NIDDK (1UC4DK104208) and Diabetes Research Institute Foundation.

LBP.39

Metabolomic profiling of breast cancer-derived extracellular vesicles: Metabolic reprograming by interferon-gamma

Hiroko Tadokoro[1], Ryuhei Kudo[2], Akiyoshi Hirayama[2], Yusuke Yoshioka[3], Masahiro Sugimoto[2] and Takahiro Ochiya[3]

[1]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute; [2]Institute for Advanced Biosciences Keio University, Tokyo, Japan; [3]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Japan

Introduction: Since some studies reveal that immune cells upon activation show distinct metabolic changes that impact their immune functions, it is focused to whether immune cells also undergo metabolic reprogramming in cancer and how this might affect their contribution in cancer progression. EVs contain various molecular constituents such as proteins, nucleic acid, and metabolites. However, the functions of metabolites in EVs remain largely unknown. Indoleamine-2,3-dioxygenase (IDO), tryptophan catabolic enzyme, is constitutively expressed in tumor and it is assumed that it serves as an immune-escape mechanism. In some cancer, IDO expression appears to be induced by cytokines, such as interferon (IFN)-gamma. Cancer-derived extracellular vesicles (EVs) also contribute to the neutralization of the anti-cancer immune response. Therefore, the purpose of this study is to identify cancer metabolites which are associated with immunosuppressive functions of the breast cancer cells-derived EVs in the presence or absence of IFN-gamma.
Methods: Metabolomic analysis of cell and EVs are performed on breast cancer cell lines, MDA-MB-231-D3H2LN (D3H2LN). D3H2LN cultured in the presence and absence of IFN-gamma. EVs were purified from cell supernatant by ultracentrifugation. EV samples were then washed with PBS twice for metabolomics analysis. Next, methanol containing internal standard was added to the sample. The metabolomic analysis was performed by CE-TOFMS and IC/LC-QE.
Results: Based on the analysis by CE-TOFMS, we found that cells contain the 95 metabolites (Positive ionization mode (Pos): 45, Negative ionization mode (Neg): 50). The 11 metabolites (Pos: 9, Neg: 2) were detected to be a higher amount in D3H2LN cell cultured in the presence of IFN-gamma and the 7 metabolites (Pos: 4, Neg: 3) were significantly a higher amount in D3H2LN cells cultured in the absence of IFN-gamma.
Summary/Conclusion: IFN-gamma induced metabolic changes in the breast cancer cell. Some metabolites are characteristic in D3H2LN cell cultured in the presence of IFN-gamma.

LBP.40

Outer membrane vesicles derived from Escherichia coli mediate neutrophil infiltration into the lungs via IL-8 release from endothelial cells

Nhung Thi Hong. Dinh[1], Yae Jin Yoon[2], Ji Hyun Kim[2], Hyun Taek Park[1], Gyeongyun Go[1], Changjin Lee[2] and Yong Song Gho[1]

[1]POSTECH; [2]Postech

Introduction: Outer membrane vesicles (OMVs) are spherical proteolipid nanostructures that are constitutively secreted by Gram-negative bacteria including *E. coli*. Our previous work showed that *E. coli* OMVs stimulate strong pulmonary inflammatory response after intraperitoneal administration to mice. This immune response led to significant infiltration of neutrophils into the lungs. Thus, the mechanisms of neutrophil recruitment by *E. coli* OMVs need to be elucidated
Methods: Mice were intraperitoneally administered with *E. coli* OMVs, then immunostaining was employed to examine neutrophil infiltration into the lungs. Lung RNAs were isolated and subjected to real-time RT-PCR to measure IL-8 expression. The localization of OMVs in the lungs was identified by immunofluorescence imaging. Various types of cells were used to find the main sources of IL-8. Relevant Toll-like receptors (TLRs) and downstream signaling pathways were examined to find the mechanisms of IL-8 release
Results: Intraperitoneal administration of *E. coli* OMVs resulted in significant infiltration of neutrophils into the lungs, and IL-8 expression was significantly increased. In addition, OMVs injected co-localized with CD31-positive cells (endothelial cells) in the lung. Among various types of cells, endothelial cells were found to be the main source of IL-8 in response to OMV treatment. Among TLRs expressing on endothelial cells, TLR4 was shown as the main component in OMV recognition. IL-8 production was notably observed on HEK293 overexpressing TLR4/MD2 cells upon OMV treatment while this function was abrogated in TLR4 knock-out mice
Summary/Conclusion: Taken together, our data revealed that *E. coli* OMVs recruit neutrophils to the lung via IL-8 release from endothelial cells in TLR4-dependent manner

LBP.41

Wharton's Jelly mesenchymal stem-stromal cell suppression of T helper cell division by exosomes is mediated by membrane bound TGFβ

Sarah Crain[1], Kristen Thane[2], Airiel Davis[2] and Andrew Hoffman[2]

[1]Tufts University Cummings School of Veterinary Medicine, MA, USA; [2]Tufts University, MA, USA

Introduction: Mesenchymal stem-stromal cells (MSC) are known to suppress activation and proliferation of $CD4^+$ T cells, and soluble transforming growth factor β (TGFβ) plays an important role in that mechanism. Immunosuppression by membrane bound TGFβ is recognized in dendritic cell and cancer associated fibroblast extracellular vesicles (EV), but this mechanism has not been documented for MSC-EV. We hypothesized that EV membrane bound TGFβ is central to the immunomodulatory mechanism of MSC.
Methods: Serum-free culture media from canine Wharton's Jelly mesenchymal stem cells (WJ-MSC: $CD44^+$ $CD90^+$ $CD34^-$ $CD45^-$ $MHCII^-$; n=6 cell lines) was collected after 48hr and EV (WJ-EV) isolated via differential ultracentrifugation. EV output was assessed using NTA. CFSE-stained peripheral blood mononuclear cells were collected from healthy dogs (n=8) exposed to concanavalin A (ConA; 5μg/ml), and co-incubated with WJ-MSC (1:10) across transwell membrane (0.4μm pore size) or WJ-MSC EV ($1:10^4$) +10μM SB431542 (TGFβR1 inhibitor) or TGFβneutralizing antibody (Ab) for 72hr. Analysis of CFSE fluorescence using FlowJo (v7.6.5) yielded $\%CD4^+$ divided.
Results: An average of 83±2% of the particle count from WJ-MSC conditioned medium were in the exosome size range (30-200nm) based on NTA. The $\%CD4^+$ division in response to ConA alone (60±17%) was significantly higher than ConA+WJ-MSC (25±11%, $P < 0.01$), ConA+WJ-EV (23±13%, $P < 0.01$), or soluble TGFβ1 alone (21±10%, $P < 0.01$). Addition of SB431542 to ConA+WJ-EV increased $CD4^+$ division to 52 ±17% ($P < 0.01$ vs ConA+WJ-EV). Addition of TGFβ Ab to ConA+WJ-EV at 0.1, 1, or 10μg/ml resulted in $CD4^+$ division of 58±14%, 60±16%, and 58±10% ($P < 0.01$ vs ConA+WJ-EV), respectively.
Summary/Conclusion: These data demonstrate that mitogen induced T cell proliferation, markedly suppressed by WJ-EV, is mediated in part by membrane bound TGFβ. Further research is necessary to determine the signaling pathway of EV-derived TGFβ.
Funding: Shipley Foundation

Satellite Event
ISEV-ISAC-ISTH Workshop
Room: Metropolitan Ballroom West and Centre 6:30–8:00 p.m.

Scientific Program ISEV2017
Saturday, May 20, 2017

Meet the Expert Morning Sessions:
Room: Metropolitan Ballroom West and Centre
MTE- Session IV: In vivo imaging-based analysis of EV-biological activity
Chair: *Eva-Maria Albers* 7:45–8:45 a.m.
Speakers: *Takahiro Ochiya and Charles Lai*

Room: Metropolitan Ballroom East
MTE- Session V: Vesicular and non-vesicular pathways of extracellular RNA release
Chair: *Esther Nolte-t Hoen* 7:45–8:45 a.m.
Speakers: *Alissa Weaver and Muneesh Tewari*

Room: Harbour Ballroom
MTE-Session VI: EV-mediated parasite-host interactions
Chair: *Ana Claudia Torrecilhas* 7:45–8:45 a.m.
Speakers: *Rodrigo Soares and Martin Olivier*

Oral Sessions
Room: Metropolitan Ballroom West and Centre
Symposium Session 19 – EVs in Tumour Immunity and Angiogenesis
Chairs: Carol Parent and Janusz Rak 9:00–10:00 a.m.

OS19.01

Release of endothelial cell-associated VEGFR2 during TGF-beta modulated angiogenesis in vitro

Alicia M. Viloria-Petit[1], Mai Jarad[1], Elizabeth Kuczynski[1], Jodi Morrison[1], Laura Montermini[2], Dong-Sic Choi[2], Janusz Rak[2] and Brenda Coomber[1]

[1]Department of Biomedical Sciences, University of Guelph, Ontario, Canada; [2]The Research Institute of the McGill University Health Center, Quebec, Canada

Introduction: Sprouting angiogenesis is regulated by soluble factors, principally vascular endothelial growth factor (VEGF), and via bidirectional signalling through the Jagged/Notch system, leading to assignment of tip cell and stalk cell identity. Transforming growth factor beta (TGFβ) can either stimulate or inhibit angiogenesis via its differential surface receptor signalling.
Methods: Using immunoblotting and qRT-PCR we evaluated changes in expression of angiogenic signalling receptors in bovine aortic endothelial cells exposed to TGFβ1, and correlated these changes to endothelial cord formation on Matrigel. The extracellular vesicles (EVs) in the conditioned media were assessed via particle tracking and proteomic analysis, following EV purification by ultracentrifugation at 100,000*g*.
Results: TGFβ1 induced a dose dependent inhibition of cord formation, maximal at 5.0 ng/ml. This occurred via ALK5-dependent pathways and was accompanied by significant upregulation of the TGFβ co-receptor endoglin, and SMAD2 phosphorylation, but no alteration in SMAD 1/5 activation. TGFβ1 also induced ALK5-dependent downregulation of Notch1 but not of its ligand delta-like ligand 4 (Dll4). Cell associated VEGFR2 (but not VEGFR1) was significantly downregulated and accompanied by reciprocal upregulation of VEGFR2 in conditioned medium. qRT-PCR analysis revealed that this soluble VEGFR2 was not generated by a selective shift in mRNA isoform transcription. This VEGFR2 was full-length protein and was associated with increased soluble HSP-90, consistent with shedding of EVs. Particle tracking and proteomic analysis indicate modulation of EV production and cargo by TGFβ1.
Conclusions: Our results suggest that angiogenesis-associated changes in endothelial cells exposed to TGFβ1 might be mediated, at least in part, by the release of key mediators of angiogenic signals, including VEGFR2, into the extracellular environment. The biological significance of this remains to be determined.

OS19.02

Mutant p53 cancers reprogram tumour associated macrophages via exosomal miR-1246

Tomer Cooks[1] and Curtis C. Harris[2]

[1]National Cancer Institute, NIH; [2]Lab of Human Carcinogenesis, NCI

Introduction: TP53 mutants are involved in the pathogenesis of most solid tumours and are known to gain oncogenic functions distinct from their original wild-type form. The existence of such gain-of-function (GOF) activities is supported by ample evidence, however only in a cell-autonomous fashion. Since tumour-associated macrophages (TAM) are also a hallmark of solid tumours typically correlated with poor prognosis, we investigated the link between mutations in the TP53 gene (mutp53) occurring in epithelial tumour cells and the formation of a surrounding TAM population *in situ*.
Methods: By designing a co-culture system we incubated human primary monocytes together with colorectal cancer (CRC) cells differing in their p53 status. Relevant macrophages markers were evaluated on RNA level and protein level. In addition, co-cultured macrophages were subjected to various functional assays (phagocytosis, migration, and invasion). In attempt to confirm clinical relevance, samples from a cohort of human CRC patients were analysed using genomic and immunohistochemical methods. To identify the interaction between the tumour cells and the macrophages, we isolated exosomes from the CRC cells and subjected them to a Nanostring analysis to learn about their microRNAs composition.
Results: In this study, we discovered that mutp53 exerts a non-cell-autonomous effect over neighbouring macrophages by using specific microRNAs (miRs) which are shuttled through an exosomal transfer resulting with a phenotype change of the affected macrophages. Mutp53-specific exosomes containing cargoes such as miR-1246 were shown to be used by macrophages at the receiving end, thus promoting the formation of TAM subset also observed in surgical specimens resected from cancer patients.
Conclusions: Mutp53-reprogammed TAM favour anti-inflammatory immunosuppression with increased activity of TGF-β. These findings, observed also in colon cancer patients, strongly support a microenvironmental GOF role for mutp53 in actively engaging the immune system to promote cancer progression and metastasis.

OS19.03

Determining the role of key regulators of apoptotic cell disassembly in cell clearance

Rochelle Tixeira[1], Christina Nedeva[1], Georgia Atkin-Smith[1], Thanh Kha Phan[1], Hamsa Puthalakath[1], Marco Herold[2], Mark Hulett[1] and Ivan Poon[1]

[1]La Trobe Institute for Molecular Science, Melbourne, Australia; [2]The Walter and Eliza Hall Institute, Parkville, Australia

Introduction: Apoptosis is a key process in maintaining homeostasis. Efficient clearance of apoptotic cells is necessary, as failure in this process is linked to various disorders including autoimmune, inflammation and cancer. In order to achieve timely clearance, apoptotic cells undergo regulated disassembly into smaller membrane bound vesicles called apoptotic bodies. Apoptotic cell disassembly (ACD) involves a series of morphological changes such as membrane blebbing, followed by string like membrane extensions termed apoptopodia, and cell fragmentation to generate apoptotic bodies. Drug based assays have shown Rho kinase 1 (ROCK1) and P21 activated kinase 2 (PAK2) are involved in membrane blebbing, while membrane protein Pannexin 1 (PANX1) is a negative regulator of apoptopodia and apoptotic body formation. While the need for efficient clearance is necessary, the role of these key regulators in ACD and their implication to cell clearance is not well understood. Moreover, understanding the clearance of apoptotic cells and bodies may provide key insights in linked diseases.
Methods: Using CRISPR gene editing technology in human Jurkat T cells as a model, gene disruptions were introduced in *ROCK1, PAK2* and *PANX1* leading to a loss of protein expression. Clonal populations showing loss of ROCK1, PAK2 and PANX1 were obtained and subject to apoptosis using UV radiation and anti-Fas. Apoptotic cells were characterised for morphology and disassembly by differential interference microscopy and flow cytometry. To determine the implications of ACD on clearance, these knockout cell clones were subject to engulfment assay using professional phagocytes namely macrophages and immature dendritic cells.
Results: ROCK1 is important for ACD as loss of ROCK1 expression in Jurkat T cells lead to impairment in both membrane blebbing and apoptotic body formation while loss of PAK2 did not affect ACD as compared to control. Cells lacking PANX1 showed marked increase in apoptotic body formation. Moreover, both macrophages and immature

dendritic cells show a preference for apoptotic bodies over apoptotic cells.
Summary: These findings suggest that apoptotic cell disassembly plays a vital role in cell clearance, whereby the formation of apoptotic bodies allows for efficient clearance by both dendritic cells and macrophages.

OS19.04

Proteomic analysis of exosomes derived from serum and cells in non–small cell lung cancer

Si-Hua Qin[1], Yong Xu[2], Taixue An[3], Yue-Ting Tang[4], Yiyao Huang[1] and Lei Zheng[3]

[1]Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangdong, China; [2]Southern Medical University Affiliated Nanfang Hospital, Guangdong, China; [3]Department of Laboratory Medicine, Southern Medical University Affiliated Nanfang Hospital, Guangdong, China; [4]Department of Clinical Laboratory, Zhongnan Hospital, Wuhan University, Hubei, China

Introduction: Exosomes are small (30–100 nm) membrane vesicles can mediate intercellular communication via transfer of proteins and other biological molecules. A number of exosomal proteins are reported as diagnostic, prognostic, or even therapeutic biomarkers in cancer patients.
Method: We employed a mass spectrometry (LC-MS/MS) quantitative proteomics strategy to examine the different exosomal proteins expression in non-small cell lung cancer (NSCLC). Exosomes, isolated from not only the pooled serum of 8 patients with NSCLC (stages I and II), 8 patients with NSCLC (stages III and IV) and 12 normal volunteers, but also the cell culture medium of an immortalised normal bronchial epithelial cell line 16HBE and a NSCLC cell line A549,were separated by ultracentrifugation. Written informed consents were obtained from all patients and normal volunteers.
Result: 696 and 1811 exosomal proteins were identified in three pooled serum and two cell lines. Compared with the SPEs of normal volunteers, we found 42 proteins upregulated and 54 proteins downregulated in the NSCLC patients, and 93 proteins were only detected in the NSCLC patients. Then 26 proteins were unregulated and 26 proteins were downregulated in the NSCLC (stages III and IV) patients compared with the SPEs of NSCLC (stages I and II) patients. The differential proteins profile associated with NSCLC exosomes that suggested a role these vesicles have in the progression of lung carcinogenesis, as well as identified several novel candidates that could be utilised as a multi-marker protein panel in a diagnostic or prognostic platform for NSCLC. Next, we found 66 proteins upregulated and 62 proteins downregulated in exosomes derived from two cell lines, 519 proteins were only identified in one cell line. Differential proteins were associated with signalling pathway, including Wnt, VEGF, PI3K-Ak, mTOR and ErbB, especially hedgehog signalling pathway were enriched in NSCLC. It also enriched in pentose phosphate pathway and amino sugar and nucleotide sugar metabolism which probably play a significant role in cancer progression.
Conclusion: The investigation of the NSCLC exosomal proteome has identified enriched protein cargo that may contribute to lung cancer progression, which may have potential clinical implications in biomarker development for patients with NSCLC.

Room: Metropolitan Ballroom East
Symposium Session 20 – EVs in Stem Cell and Cardiovascular Biology
Chairs: Costanza Emanueli and Uta Erdbruegger 9:00–10:00 a.m.

OS20.01

Exosomes as a vector for Wnt7a systemic treatment in Duchenne Muscular Dystrophy

Uxia Gurriaran[1,2], Fan Xiao[1,2] and Michael A. Rudnicki[1,2]

[1]Sprott Centre for Stem Cell Research, Ottawa Hospital Research Institute, Ottawa, Canada; [2]Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Canada

Introduction: Duchenne muscular dystrophy (DMD) is a genetic myopathy characterised by the lack of dystrophin, evoking skeletal muscle debilitation and ultimately death. To date, no successful therapies exist to cure DMD. Our group discovered that Wnt7a, a secreted glycoprotein, represents an intrinsic mechanism for skeletal muscle regeneration. Local muscle injection of Wnt7a into dystrophic mice restores muscle function by stimulating muscle regeneration. However, given its hydrophobic nature, Wnt7a cannot be delivered systemically. Our goal is to overcome this limitation by testing the suitability of exosomes as a vector for systemic delivery of Wnt7a.
Methodology: Exosomes were isolated from human transfected cells using a novel method designed by our laboratory that allows for the separation of exosomal secreted Wnt7a and secreted non-exosomal Wnt7a. Muscle regenerative effect of exosomal Wnt7a was tested by assaying exosome uptake in mice primary myoblasts, expansion of muscle stem cells cultured *ex vivo* on cultured myofibers, and induction of myotube and myofiber hypertrophy.
Results: After transfection of expression plasmids into human cells, Wnt7a is specifically secreted on the exosomal surface. Unexpectedly, Wnt7a exosomal secretion is not impaired upon specific mutation of the pamitoylation sites in Wnt7a. Therefore, unlike other Wnts, pamitoylation is not required for Wnt7a exosomal secretion. Furthermore, exosomal Wnt7a generated from human cells readily stimulates murine satellite cell symmetric expansion, motility, and myofiber hypertrophy, eliciting the full biological response of Wnt7a in muscle.
Conclusions: Our experiments demonstrate that Wnt7a is efficiently delivered through exosomes into myogenic cells where it elicits the full regenerative response in skeletal muscle. Moreover, we found that pamitoylation is not required for secretion of Wnt7a in exosomes. This finding suggests that different mechanisms are involved in short- versus long-range Wnt signalling. Taken together, our data indicates that delivery of Wnt7a via exosomes represents a promising therapeutic avenue for system delivery for treating DMD.

OS20.02

Angiogenic mechanisms of human CD34+ stem cell exosomes in the repair of ischemic heart

Yaxuan Liang[1], Prabhu Mathiyalagan[1], Sol Misener[2], Douglas Losordo[3] and Susmita Sahoo[1]

[1]Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai, New York, USA; [2]Feinberg Cardiovascular Research Institute, Feinberg School of Medicine, Northwestern University, NY, USA; [3]Caladrius Biosciences

Introduction: Locally transplanted human $CD34^+$ stem cells have been shown to improve exercise tolerance in patients with myocardial ischemia and promote angiogenesis in animal models. In an earlier study, first of its kind, we have demonstrated that $CD34^+$ cells secrete exosomes (CD34Exo) that constitute a critical component of the pro-angiogenic paracrine activity of the cells. Here, we investigated the mechanisms of CD34Exo-mediated repair of the ischemic myocardium and therapeutic angiogenesis by studying their miRNA content and uptake.
Methods and Results: Using a murine model of myocardial ischemia we found that CD34Exo replicated the therapeutic activity of their parent cells by significantly improving myocardial ischemia (ejection fraction, 42 ± 4 vs. 22 ± 6%, capillary density, 113 ± 7 vs. 66 ± 6/HPF, fibrosis, 27 ± 2 vs. 48 ± 7%, $p < 0.05$, $n = 7–12$), compred with PBS control. Knocking down miR-126 from CD34exo abolished their angiogenic activity and beneficial function both in vitro and in vivo. Injection of CD34Exo increased miR-126 levels in mouse ischemic heart, but did not affect the endogenous synthesis of miR-126 suggesting a direct transfer of stable and functional exosomal miR-126. miR-126 enhanced angiogenesis by suppressing the expression of its known target, SPRED1 and simultaneously modulating the expression of genes involved in angiogenic pathways such as VEGF, ANG1, ANG2 etc. Interestingly, CD34Exo, when treated to ischemic hearts, was most efficiently internalised by endothelial cells relative to smooth muscle cells and fibroblasts demonstrating a direct role of stem cell-derived exosomes on mouse endothelium at the cellular level.
Conclusion: Collectively, our results have demonstrated a novel mechanism by which cell-free CD34Exo mediates ischemic tissue repair via beneficial angiogenesis. Exosome-shuttled angiomiRs may signify amplification of stem cell function and may explain the angiogenic and therapeutic benefits associated with $CD34^+$ stem cell therapy.Trafficking studies using confocal imaging and flow cytometry analyses revealed that CD34Exo was selectively internalised by endothelial cells and cardiomyocytes relative to fibroblasts in the CD34Exo-injected ischemic hearts. MicroRNA expression profiling and Taqman assays indicated that CD34Exo are significantly enriched with pro-angiogenic miRNAs such as miR126. CD34Exo injection induced the expression of miR126 and several pro-angiogenic mRNAs in mouse ischemic myocardium, suggesting a direct transfer of miR126. CD34Exo lacking in miR126 had decreased angiogenic and therapeutic activity both *in vitro* and *in vivo* indicating that miR126 was important for CD34Exo function.

OS20.03

Mesenchymal stem cells and their secreted exosomes exert therapeutic effects in Duchenne muscular dystrophy

Ariel Bier[1], Peter Bernstein[1], Simona Cazacu[2], Amir Dori[3] and Chaya Brodie[4]

[1]Bar-Ilan University, Israel; [2]Henry Ford Health Systems, Detroit, MI, USA; [3]Sheba Medical Centre, Israel; [4]Faculty of Life Sciences Bar-Ilan University, Israel and Neurosurgery Department, Henry Ford Health Systems, Detroit, MI, USA

Duchenne muscular dystrophy (DMD) is a progressive lethal, X-linked disease of skeletal and cardiac muscles caused by mutation of the dystrophin gene, which leads to muscle degeneration. Cell therapy using different cell types has been considered a potential therapeutic approach for the treatment of DMD. Mesenchymal stromal cells (MSCs) are obtained from autologous bone marrow and adipose tissues or from allogeneic placenta and umbilical cord. The safety and therapeutic impact of MSCs have been demonstrated in pre-clinical and clinical studies and are attributed to paracrine effects that are partly mediated by extracellular vesicles. Here, we studied the therapeutic effects of MSCs and their secreted exosomes using human *in vitro* disease models of skeletal muscle cultures derived from healthy and Duchenne patients and MDX mice. Treatment of satellite cells with conditioned media or exosomes secreted by MSCs increased the proliferation and generation of $PAX7^+$/$MyoD^+$ cells and the differentiation of human myoblasts from both healthy and DMD patients. MSCs from different sources exerted differential effects on the function of the muscle cells. Secretome and RNA sequencing analysis of the MSC-derived exosomes revealed specific cytokines and clusters of miRNAs and long non-coding RNAs that were associated with anti-inflammatory and pro-regenerative activities in muscle cells. Using novel quantitative miRNA reporters, we demonstrated that MSC-derived exosomes delivered both endogenous and

exogenous miRNAs to satellite cells and myoblasts. Intramuscular implantation of MSCs to MDX mice resulted in decreased tissue fibrosis and CK level, increased differentiation of satellite cells, expression of utrophin and motor function. Imaging analyses using labelled MSCs and exosomes, demonstrated their localisation in the muscle tissues up to 4 weeks. These results demonstrate that MSCs and their secreted exosomes have important clinical applications in cell therapy of DMD partly via the targeted delivery of therapeutic non-coding RNAs.

OS20.04

Exosomes and microparticles released by mesenchymal stem cells exert a chondroprotective effect in osteoarthritis

Stella Cosenza[1], Karine Toupet[1], Claire Bony[1], Olivier P. Blanc-brude[2], Christian Jorgensen[3] and Daniele Noel[4]

[1]Inserm u1183; [2]INSERM UMR970 – Paris Cardiovascular Research Centre (ParCC), Paris, France; [3]CHU Montpellier-University of Montpellier, France; [4]Inserm

Introduction: Mesenchymal stem cells (MSC) are multipotent cells that possess regenerative functions that are of interest for in osteoarticular diseases such as osteoarthritis (OA). These functions are thought to be primarily mediated by mediators released within extracellular vesicles (EVs). EVs consist of exosomes, microparticles and apoptotic bodies that mirror the effect of parental cells but little is known about their respective role . The aim of this study was to compare the immunomodulatory effects of exosomes and microparticles secreted by MSCs.

Methods: EV subsets were isolated from murine primary MSCs by ultracentrifugation. Size and structure were evaluated by dynamic light scattering and electron microscopy. Expression of membrane and endosomal markers was tested by flow cytometry or western blot. Proliferation of murine splenocytes was quantified after 72 h of incubation with EVs after CFSE-labelling. Phenotypic analysis of T lymphocyte subpopulations was also performed by flow cytometry. *In vivo*, EVs were injected in the knee joint in the collagenase-induced osteoarthritis (CIOA) model and histological score was performed.

Results: MSC-derived exosomes were less than 120 nm in diameter and expressed CD9, CD81 and TSG101 while microparticles were around 400 nm in diameter and expressed CD29, CD44 and Sca1 MSC markers. *In vitro* functional analysis indicated that addition of microparticles or exosomes in proliferative assays inhibited the proliferation of total splenocytes in a dose-dependent manner. Analysis of T cell subpopulations revealed a decrease in $CD8^{+}IFN\gamma^{+}$ lymphocytes and an increase in both $CD4^{+}IL10^{+}$ Tr1 and $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells. This immunomodulatory function of EVs was also observed *in vivo* in the CIOA model with a significantly reduced osteoarthritic score on histological sections.

Conclusion: Our *in vitro* data indicated that the immunosuppressive effect of MSCs is in part mediated by exosomes and microparticles. These vesicles were shown *in vivo* to play a major role in MSC-mediated therapeutic effect by reducing osteoarthritic symptoms.

Room: Harbour Ballroom
Symposium Session 21 – Milk EVs
Chairs: Martinjn van Herwijnen and Patrick Provost 9:00–10:00 a.m.

OS21.01

Milk exosomes enhance anti-proliferative and anti-cancer activities of berry anthocyanidins against multiple human cancers

Ramesh C. Gupta[1], Jeyaprakash Jeyabalan[2], Ashley Mudd[3], Ashish Kumar Agrawal[2], Al-Hassan Kyakulaga[3], Wendy Spencer[4], Manicka V. Vadhanam[2], Farrukh Aqil[5] and Radha Munagala[5]

[1]Department of Pharmacology and Toxicology and JG Brown Cancer Center, University of Louisville, KY, USA; [2]JG Brown Cancer Center, University of Louisville, KY, USA; [3]Department of Pharmacology and Toxicology, University of Louisville, KY, USA; [4]3P Biotechnologies, Inc., Louisville, KY, USA; [5]Department of Medicine and JG Brown Cancer Center, University of Louisville KY, USA

Introduction: Berries have been reported with beneficial effects against various diseases including cancer, and anthocyanidins (Anthos) are considered presumptive active components. However, bioactivities of Anthos are compromised due to their instability and poor oral bioavailability. Here we report that therapeutic efficacy of Anthos can be enhanced when used as exosomal formulation (ExoAnthos), and that it lacks toxicity.
Methods: ExoAnthos was prepared by incubation of bilberry-derived Anthos with bovine milk exosomes, and harvesting of ExoAnthos by ultracentrifugation which was then analysed for particle size by zetasizer and AFM. Antiproliferative potential of ExoAnthos was determined by MTT assay against various human cancer cells (lung, breast, prostate, pancreas, and colon). Anti-cancer activity of ExoAnthos was determined against lung tumour xenograft in nude mice. Toxicity was determined in wild-type mice treated orally with the Anthos, Exo and ExoAnthos for 3 weeks.
Results: Significant loading of Anthos was observed in the exosomes (Exo) presumably due to hydrophobic interactions as revealed by a substantial quenching of fluorescence inherent of the native Exo proteins. Particle size of ExoAnthos was essentially unchanged vs. Exo (79 vs. 83 nm). HPLC analysis of ExoAnthos stored at −80°C showed no change in the drug load for up to 4 weeks. ExoAnthos showed 5- to 50-fold reduction in the IC_{50}values of ExoAnthos vs. Anthos presumably due to higher cell up take and stability. Anthos (10 mg/kg) given orally three times a week showed a slight but insignificant inhibition of the tumour growth. However, ExoAnthos even at a lower dose (5 mg/kg) showed significant inhibition of lung tumour xenograft (>40% reduction, $p < 0.01$). Analysis of blood and the plasma showed no effect on the liver and kidney function enzymes and hematopoietic parameters following treatment with ExoAnthos, Anthos or Exo indicating these agents were well tolerated.
Conclusion: Our data demonstrate significantly enhanced antiproliferative and anticancer activities of the berry Anthos when embedded in the exosomes, and suggest that milk exosomes may serve as an excellent nano carrier for plant bioactives that encounter stability and oral bioavailability issues.
Financial support: Duggan Endowment and Helmsley Trust Fund.

OS21.02

Characterisation of extracellular vesicles with milk fat globule membrane-like properties that carry most microRNAs in commercial dairy cow milk

Benmoussa Abderrahim[1], Ly Sophia[2], Shan Si Ting[2], Jonathan Laugier[2], Eric Boilard[2], Gilbert Caroline[2] and Patrick Provost[2]

[1]Centre de Recherche du CHU de Québec /Pavillon CHUL – Université Laval, Quebec, Canada; [2]Department of Microbiology-Infectious Disease and Immunity and Faculty of Medicine, Université Laval, Quebec, Canada

Introduction: MicroRNAs are short (~22 nucleotides), non-coding RNAs that play an essential role in post-transcriptional gene regulation. Found in several biological fluids, including milk, they are often associated with extracellular vesicles (EVs), like exosomes. In a previous study, we found that commercial dairy cow milk microRNAs resist digestion *in vitro*. Surprisingly, we observed thatmost of them sediment at low centrifugation speed, thereby challenging their association with exosomes in commercial milk.
Methods: We used differential ultracentrifugation and iodixanol density gradient (IDG) to isolate milk EVs, which we analysed for microRNA enrichment by reverse transcription and quantitative polymerase chain reaction (RT-qPCR) and for EV-associated proteins by western blot. We further characterised these EVs by density measurements, fluorescence RNA labelling, mass spectrometry (LC-MS/MS), dynamic light scattering (DLS), flow cytometry, transmission electron microscopy (TEM) and proteinase K assay.
Results: We found no correlation between bta-miR-223 and bta-miR-125b and exosome-associated proteins found in low speed ultracentrifugation pellets (i.e. 12,000*g* and 35,000*g*), but a positive correlation ($p < 0.05$) between bta-miR-125b and xanthine dehydrogenase (XDH). Two IDG fractions were highly enriched in double stranded RNAs and microRNAs, contained several exosome-associated proteins and most of the exosome-like EVs found in these gradients. However, proteinase K assay and subsequent LC-MS/MS analysis challenged the exosome nature of these EVs, as all exosome-enriched proteins were digested during the assay and these digested EVs were found to contain milk fat globule membrane (MFGM)-enriched proteins, including immunomodulatory XDH, butyrophilin 1A1 (BTN1A1), mucin (MUC-1) and lactadherin (MFG-E8).
Conclusion: Our results suggest the presence of exosome-like EVs with MFGM-like properties in commercial milk and their association with the majority of milk microRNAs.
Considering their resistance to proteinase K digestion and bioaccessibility *in vitro*, these EVs may contribute to interspecies transfer of dietary microRNAs and immune regulation by milk EVs, which require further investigations.
Financial support: CIHR grants No. 319618 and 327522 (to P.P.).

OS21.03

Tracing cellular origin of human exosomes using multiplex proximity extension assay

Pia Larssen[1], Lotta Wik[2], Paulo Czarnewski[1], Maria Eldh[1], Liza Löf[2], Göran Ronquist[2], Louise Dubois[2], Eva Freyhult[2], Caroline Gallant[2], Johan Oelrich[2], Anders Larsson[2], Gunnar Ronquist[2], Eduardo Villablanca[1], Ulf Landegren[2], Masood Kamali-Moghaddam[2] and Susanne Gabrielsson[3]

[1]Karolinska Institute, Solna, Sweden; [2]Uppsala University, Uppsala, Sweden; [3]Immunology and Allergy Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden

Extracellular vesicles (EVs) are membrane-coated objects such as exosomes and microvesicles, released by many cell-types. Their presence in body fluids and the variable surface composition and content render them attractive potential biomarkers. The ability to determine their cellular origin could greatly move the field forward. We used multiplex proximity extension assays (PEA) to identify with high specificity and sensitivity the protein profiles of exosomes of different origins, including seven cell lines and two different body fluids. By comparing cells and exosomes, and after appropriate data filtering, we successfully identified the cells originating the exosomes. Furthermore, human milk EVs and prostasomes released from prostate acinar cells clustered with cell lines from breast and prostate tissue, respectively. Milk exosomes uniquely expressed CXCL5, MIA and KLK6, while prostasomes carried NKX31, GSTP1 and SRC, highlighting that EVs originitating from different origins have different properties. In conclusion, PEA provides a

powerful protein screening tool in exosome research, for purposes of identifying the cell source of exosomes, or new biomarkers in diseases such as cancer and inflammation.

OS21.04

Biological activities of extracellular vesicles and their cargos from bovine milk in non-bovine species

Sonia Manca, Fang Zhou, Mahrou Sadri, Jiang Shu, Jacob Jarecke, Ana Aguilar-Lozano, Amy Leiferman, Ryan Grove, Henry Paz, Jiri Adamec, Samodha Fernando, Juan Cui and Janos Zempleni

University of Nebraska-Lincoln, NE, USA

Background: Extracellular vesicles (EVs), and in particular exosomes, play important roles in cell-to-cell communication, facilitated by the transfer of nucleic acids, lipids, and proteins from donor cells to recipient cells. Exosomes in bovine milk are absorbed by endocytosis and deliver their cargos to circulating cells and tissues in non-bovine species. The phenotypes of dietary exosome depletion are unknown.
Hypothesis: Bovine milk exosomes and their cargos have biological activities in non-bovine species.
Objectives: (1) Assess the bioavailability and distribution of bovine milk exosomes and RNA cargos in mice. (2) Assess whether milk exosome-defined diets elicit phenotypes in mice. 3) Assess whether milk exosomes alter the gut microbiome.
Methods: In bioavailability studies, milk exosomes and RNA cargos were labelled with DiR and Exo-Red, respectively, and the tissue distribution was assessed using iBox and Licor imagers following oral exosome administration. In phenotype and microbiome studies, C57BL/6 mice were fed AIN93G-based, milk exosome-defined diets for up to 42 weeks, starting at weaning (3 wk). At ages 7, 15 and 45 weeks, phenotypes were assessed using a hypothesis-generating, unbiased phenotype screen.
Results: While the majority of exosomes accumulated in spleen and liver, their RNA cargos localised to brain and kidney. A fraction of exosomes was not absorbed and entered the large intestine. Phenotypes of dietary milk exosome depletion included an about 50% decrease in litter size, up to 1900% and 144% increases in liver amino acids and purines, respectively, and 150% and 200% percent impaired spatial memory and kainic acid survival times. Depending on sex and age, between 200 and 500 "strains" of microorganisms (operational taxonomic units) were differentially expressed in mice fed an exosome-depleted diet compared with exosome-sufficient controls.
Conclusions: Bovine milk exosomes and their cargos are bioavailable and accumulate in distinct tissues. Exosome-defined diets elicit strong phenotypes, and changes in the gut microbiome might contribute towards these phenotypes.

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Room: Metropolitan Ballroom West and Centre
Plenary Session 03 – From Extracellular Vesicles to Coordinated Behaviour of Cellular Populations
Chairs: *Marca Wauben, Kenneth W Witwer* 10:30–11:30 a.m.
Speakers: *Steven Lindow, PhD* (University of California, Berkeley, CA, United States)
Novel Role of Quorum Sensing-regulated Extracellular Vesicles in Intercellular Movement and Virulence in the Plant Pathogenic Bacterium Xylella Fastidiosa
Neta Regev-Rudzki, PhD (Weizmann Institute of Science, Rehovot, Israel)

Room: Metropolitan Ballroom West and Centre
ISEV General Assembly 11:30–12:30 p.m.

Oral Sessions
Room: Metropolitan Ballroom West and Centre
Symposium Session 22 – EV Mediated Communication Between Host and Microorganisms
Chairs: Patricia Xander and Ana Claudia Torrecilhas 1:30–3:00 p.m.

OS22.01

The role of extracellular vesicles (MalaEx) from the commensal yeast Malassezia sympodialis in atopic eczema

Helen Vallhov[1], Henrik Johansson[2], Ulf Gehrmann[3], Tina Holm[3], Janne Lehtiö[2] and Annika Scheynius[1]

[1]Department of Clinical Science and Education, Karolinska Institutet, and Sachs' Children and Youth Hospital, Södersjukhuset, Stockholm, Sweden; [2]Science for Life Laboratory, Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden; [3]Department of Medicine Solna, Translational Immunology Unit, Karolinska Institutet and University Hospital, Stockholm, Sweden

Introduction: Malassezia is the dominant commensal fungi in the human skin mycobiome but is also associated with common skin disorders including atopic eczema (AE). More than 50% of AE-patients have specific IgE and T-cell reactivity towards *Malassezia sympodialis*, which is one of the most frequently isolated species from both AE patients and healthy individuals. Malassezia releases nanosized exosome-like vesicles, designated MalaEx, which carry allergens and can induce inflammatory cytokine responses (1). Recently, we detected several small RNAs in MalaEx and interestingly, bioinformatic analyses indicated that MalaEx have an RNAi-independent route for biogenesis (2). We did not find any significant difference concerning the levels of these RNAs or the production and the morphology of the MalaEx when comparing MalaEx, which have been isolated from *M. sympodialis* cultured at normal skin pH versus the higher pH present on the skin of AE patients. Our aim is now to further understand how MalaEx is involved in host-microbe interactions, by comparing protein content of MalaEx and the whole yeast cells, and by investigating interactions of MalaEx with cells in the skin.
Methods: MalaEx are collected from *M. sympodialis* cultures by serial ultracentrifugation and when needed by sucrose gradient. The particle size is estimated by NanoSight and transmission electron microscopy (TEM). The protein content of MalaEx ant the whole yeast cells is assessed with quantitative proteomic analysis. Human primary cells are isolated from skin taken care after cosmetic surgery and cultured together with MalaEx.
Results: We have identified 2714 proteins in whole yeast cells and approximately 300 in MalaEx. 34 proteins are enriched in MalaEx and among those two of the major *M. sympodialis* allergens, Mala s 1 and s 7. Preliminary functional experiments suggest an active binding of MalaEx to human keratinocytes using confocal microscopy.
Conclusion: Our results support an active involvement of MalaEx in host-microbe interactions, by binding to host cells, and by the spreading of allergens, thereby contributing to the allergic inflammation. By understanding the role of MalaEx in the sensitisation and maintenance phases of AE, novel prevention strategies and potential therapeutic targets may arise.

References

1. Gehrmann U et al., *PLoS One*. 2011; 6(7):e21480.
2. Rayner S et al., *Sci Rep*. 2017; 7:39742.

OS22.02

Vesicle-mediated cross-species RNA interference between the gastrointestinal nematode *Heligmosomoides polygyrus* and its mouse host

Franklin W.N. Chow[1], Cesare Ovando Vazquez[2], Georgios Koutsovoulos[3], José Roberto Bermúdez Barrientos[2], Tuhin Maity[4], Mark Blaxter[3], Julie Claycomb[4], Cei Abreu-Goodger[2] and Amy H. Buck[1]

[1]Institute of Immunology and Infection Research, Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom; [2]Langebio–Cinvestav; [3]University of Edinburgh, United Kingdom; [4]University of Toronto, Canada

Introduction: Extracellular RNA has been proposed as a means of cell-to-cell communication within an organism and a mechanism of cross-species communication. We previously showed that an Argonaute protein (HpWAGO) and small RNAs (miRNAs and Y-RNAs) are secreted in extracellular vesicles produced by *Heligmosomoides polygyrus*, a gastrointestinal nematode that infects mice. Some of these miRNAs can suppress mouse immune-related host genes in a reporter assay. Here we describe the biochemical properties of the secreted Argonaute protein including its potential interaction partners inside the nematode as well as the mouse host.
Methods: Sucrose gradient purification of the ultracentrifuged excreted-secreted material from *H. polygyrus* followed by western blot analysis was used to determine the association of the nematode Argonaute protein with extracellular vesicles. Associated protein binding partners of the Argonaute protein were then identified by immunoprecipitation followed by LC MS/MS in *H. polygyrus* as well as a *C. elegans* strain in which it has been introduced (HpWAGO::GFP). Small RNA sequencing analysis was used to identify the class of Argonaute-associated RNAs in the secreted material and modified nucleotide analogues were used to label nematode RNA to examine uptake into mouse cells using CLICK chemistry.
Results: The *H. polygyrus* co-fractionates with extracellular vesicles based on sucrose gradient purification and co-fractionates with a class of secreted secondary small siRNAs based on size exclusion chromatography. Consistent with this, immunoprecipitation experiments of the Argonaute protein suggest it associates with factors involved in secondary siRNA biogenesis inside of nematodes. Most of these siRNAs come from unannotated regions within the *H. polygyrus* genome, not clearly associated with protein-coding genes. The nematode RNA can be visualised in the cytoplasm of mouse cells following incubation in transwell assays.
Conclusion: These results suggest further diversity in extracellular RNA from a nematode parasite and lay a foundation for understanding the origin of these exRNAs inside the parasite and the mechanisms by which they mediate cross-species communication through interaction with mouse genes.

OS22.03

Membrane vesicles from *Piscirickettsia salmonis* induce protective immunity and reduced disease development in an adult zebrafish model

Julia Tandberg[1], Leidy Lagos[2], Mona Gaarder[1], Petter Langlete[1], Erik Ropstad[2] and Hanne Winther-Larsen[1]

[1]University of Oslo, Norway; [2]Norwegian University of Life Sciences, Oslo, Norway

Introduction: Bacterial membrane vesicles (MVs) are 50–250 nm spherical structures secreted from the surface of many bacteria. Proteomic and biochemical characterisation has revealed that the vesicles contain a variety of bacterial components, including proteins, lipopolysaccharides, DNA and RNA. This makes MVs interesting as potential vaccine candidates, as they represent several aspects of the bacteria, but in a non-replicative form. MV-based vaccines have, furthermore, been successfully used for epidemic control in against serogroup B meningococcal disease, but there are still little known regarding the use of MV-based vaccines in other animals. We recently identified and characterised MVs from the fish pathogen *Piscirickettsia salmonis*, which showed that the isolated MVs share several similarities

with the bacteria. Thus, the present study focused on evacuating the use of MVs from *P. salmonis* as a vaccine candidate using an adult zebrafish model.
Methods: Adult zebrafish were immunised with a concentration of 20 µg MVs or phosphate buffer by i.p. injection. The fish were then challenge by i.p. injection after an immunisation period of 28 days with a challenge dose of 10^8 CFU *P. salmonis*. Serum and organ sampling for immunoblot analysis and RT-qPCR was performed 1, 14 and 28 days post-immunisation and 1, 3, 5 and 28 days post-challenge. Fish for histology was sampled at 28 days post-immunisation and 3 and 7 days post-challenge. All zebrafish experiment was approved by The Norwegian Animal Research Authority.
Results: Immunisation with MVs protected zebrafish against a lethal dose of *P. salmonis*, and histology showed a reduced formation of granulomas compared to the control group. Immunised fish also displayed an increased macrophage response and reduced inflammatory response after challenge, as well as an increased IgM response after vaccination.
Summary: Our data suggest an immunogenic potential of *P. salmonis* MVs and indicate an important immune response associated with *P. salmonis* pathogenesis and protection.

OS22.04

Extracellular vesicles released by m. tuberculosis-infected macrophages contain mycobacterial RNAs and induce Type I interferon expression in uninfected cells

Yong Cheng and Jeff Schorey

University of Notre Dame, IN, USA

Introduction: *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is an intracellular pathogenic bacterium which primarily infects pulmonary macrophages. Approximately one third of the world's population is infected with *M. tuberculosis* of which 5–10% develop active TB at some point in their lives. In 2015 this resulted in an estimated 10.4 million new active TB cases and 1.8 million deaths. Our studies aim to better understand how this pathogen intersects with our immune system with the primary focus being on the release of extracellular vesicles (EVs) and their role during an *M. tuberculosis* infection. The current study addresses the presence of mycobacterial RNA in EVs and their function as modulators of an immune response.
Methods: Next-generation sequencing (NGS) technique (Illumina MiSeq) and a subsequent RNA analysis pipeline was used to reveal mycobacterial transcript profile in exosomes isolated from the serum of mice infected with *M. tuberculosis*. Mycobacterial genetic manipulation, quantitative real-time PCR and ELISA were performed to determine *M. tuberculosis* components that contribute to the trafficking of mycobacterial transcripts into exosomes. Type I interferon (IFN-β) was measured by both quantitative RT-PCR and ELISA.
Results: Sixteen potential mycobacterial transcripts were originally identified from serum exosomes of mice infected with *M. tuberculosis* using Illumina MiSeq data. RT-PCR and DNA sequencing further determined the existence of mycobacterial transcripts in these exosomes that include *mce1B, rpoC, rv0730, rv1629* and *rv0453*. The abundance of these mycobacterial transcripts was markedly diminished in exosomes released by macrophages infected with a Δ*secA2* mutant of *M. tuberculosis* in which the *secA2* gene was inactivated by a transposon insertion. Consistent with RNA viruses, exosomes isolated from *M. tuberculosis*-infected macrophages induced a dose-dependent expression of IFN-β in primary murine macrophages.
Conclusion: *M. tuberculosis* transcripts are delivered into exosomes of host cells via a SecA2-dependent pathway, and these mycobacterial transcripts may induce expression of type I interferon in neighbouring cells, potentially increasing mycobacterial survival in TB patients.

OS22.05

Withdrawn at author's request.

OS22.06

Dysregulation of nutritional immunity during respiratory virus infection enhances *Pseudomonas aeruginosa* biofilm growth

Matthew Hendricks[1], Jeffrey Melvin[1], Yingshi Ouyangi[2], Donna Stolz[1], Yoel Sadovsky[2] and Jennifer Bomberger[1]

[1]University of Pittsburgh, PA, USA; [2]Magee Womens Research Institute, PA, USA

Clinical observations link respiratory virus infection and chronic *Pseudomonas aeruginosa* infection in chronic lung disease patients, including cystic fibrosis, but the mechanism underlying this interaction is not well understood. The development of chronic *P. aeruginosa* infections often involves the development of highly recalcitrant biofilm communities in the lung. We have recently shown that respiratory syncytial virus (RSV) co-infection significantly increases *P. aeruginosa* biofilm growth on airway epithelial cells (AECs) through a mechanism that is dependent on the induction of antiviral innate immune response and apical release of the host iron-binding protein transferrin, suggesting that RSV dysregulates nutritional immunity in the airway epithelium (1). However, the mechanism by which transferrin is released from AECs during respiratory viral infection remains undefined. We hypothesised that respiratory viral infection causes a mislocalisation of transferrin within AECs and allows its apical secretion, thereby promoting *P. aeruginosa* biofilm biogenesis. In the current study, we show that extracellular vesicles released apically from AECs during RSV co-infection enhanced *P. aeruginosa* biofilm growth. The extracellular vesicles had significantly increased levels of iron and chelation of iron from the extracellular vesicles reduced their ability to stimulate *P. aeruginosa* biofilm growth. Interestingly, RSV infection enhanced transcytosis and apical secretion of transferrin loaded onto extracellular vesicles. Together these results suggest RSV infection redirects transferrin trafficking in AECs, resulting in the loading of transferrin onto extracellular vesicles, which are secreted from AECs and can be utilised as an iron source by *P. aeruginosa* to form biofilms. Interferon signalling, which is a key component of antiviral immunity, replicates the enhanced biofilm formation observed during viral co-infection. We are currently investigating mechanisms by which interferon signalling induces transferrin packaging and secretion in extracellular vesicles to stimulate *P. aeruginosa* biofilm growth. Our data suggest a novel nutrient acquisition pathway for bacteria and provide mechanistic insight into nutritional immunity in the lung.

Reference

1. Hendricks et al., *PNAS*. 2016; ?.

Room: Metropolitan Ballroom East
Symposium Session 23 – EV-Based Cancer Biomarkers
Chairs: Aled Clayton and Lorraine O'Driscoll 1:30–3:00 p.m.

OS23.01

A novel biochip for capture and characterisation of extracellular vesicle subgroups in cancer patient plasma

Kwang J. Kwak, Hong Li and L. James Lee

Chemical and Biomolecular Engineering at Ohio State University, OH, USA

Introduction: Extracellular vesicles (EVs) are small membrane-bound fluid particles comprised of exosomes, microvesicles, apoptotic bodies and others that are released by different mechanisms from almost all cell types. The specific surface receptors provide means to sort EVs into relatively homogeneous subgroups. The most widely used antibody-driven method for isolating and characterising EVs involves the use of microfluidics or micron-sized magnetic beads for EV sorting and total RNA and protein based analysis for characterisation. These methods are tedious and can only provide average information from all sorted EVs. We have developed a facile technology termed immuno-tethered lipoplex nanoparticle (ILN) biochip.
Methods: Our ILN biochip is based on surface marker specific antibody to capture EV subgroups and cationic lipoplex nanoparticles (CLNs) containing RNA-specific molecular beacons (MBs) that can fuse with the captured EVs and detect specific RNA targets in individual EVs with a very small sample volume (e.g. 10–20 uL plasma) within 4 hours assay time. When the specific EVs are captured onto the glass chip surface by tethered antibody, the fluorescence signal from hybridisation between the MBs and target RNAs can be detected by total internal reflection fluorescence microscopy after EV-CLN fusion.
Results: We sorted malignant multiple myeloma (MM) cells (CD38+CD138+CD19-) and normal B cells (CD38-CD138-CD19+) from peripheral blood of MM patients and used our ILN biochip tethered with anti-CD38 mAb to capture and characterise the CD38+ EVs from both the MM cell secreted EVs and circulating EVs in blood plasma. For all studies, approval and consent was obtained from The Ohio State University institutional review board and in accordance with the Declaration of Helsinki. The results showed that the ILN biochip can clearly distinguish MM patients from healthy donors by upregulated miR-29b and down-regulated miR-16 expression in captured CD38+ EVs from plasma samples, much better than qRT-PCR or other methods relying on total EVs in plasma. A similar performance for chronic lymphocytic leukaemia patients was observed by CD20+ and CD37+ mAb captured EV subgroups.
Conclusion: We are extending this technology application to early detection of solid tumours such as lung cancer and pancreatic cancer.

OS23.02

Circulating microparticles as predictive biomarkers of severe complications of radiotherapy for prostate adenocarcinoma

Alexandre Ribault[1], Mohamedamine Benadjaoud[2], Claire Squiban[1], Romaric Lacroix[3], Coralie Judicone[4], Laurent Arnaud[4], Jean-Marc Simon[5], Florence Sabatier[4], Stephane Flamant[1], Marc Benderitter[2] and Radia Tamarat[2]

[1]IRSN/PRP-HOM/SRBE/LR2I; [2]IRSN/PRP-HOM/SRBE; [3]Aix-Marseille Université, VRCM, UMR-S1076, INSERM, UFR de Pharmacie, Marseille, France and Department of Haematology and Vascular Biology, CHU La Conception, APHM, Marseille, France; [4]Département d'Hématologie et de Biologie Vasculaire, CHU La Conception, Assistance Publique-Hôpitaux de Marseille; [5]Hôpital la Pitié Salpétrière, Assistance Publique-Hôpitaux de Paris, France

Introduction: Microparticles (MPs) are membrane fragments with biological activities shed from activated cells. MPs have been studied as biomarkers in several inflammatory diseases and as central players in intercellular communication. In this study we investigated the potential use of MPs as predicitive biomarkers of normal tissue complication after radiotherapy for prostate cancer.
Methods: We included 217 patients overexposed during a course of conformal 3D radiotherapy for a prostate adenocarcinoma between 2000 and 2006 in Jean MONNET hospital, Epinal, France. Their platelet-free plasma was obtained after several centrifugations then MPs were quantified and phenotyped by flow cytometry. The rectal toxicity scores following the blood sampling were collected during the routine follow-up and were tested for association with MPs using a logistic regression adjusted on several clinical confounders. Furthermore, anal canal, anterior prostate and bladder dose volume histograms (DVHs) informations were extracted for 36 patients to investigate MPs dosimetric correlations.
Results: A significant correlation was found between the number of platelet-derived MPs (PMPs) and monocyte-derived MPs (MMPs) with the range of doses up to the median exposure (40 Gy) of bladder/rectum and anterior prostate respectively. No correlation with the most elevated doses was found. Furthermore, a high level of MMPs was significantly associated to an increased risk of grade ≥ 3 rectal bleeding (OR = 1.19 [1.02–1.39] by +10 MMPs/µl, p = 0.02) and to a borderline significant risk of grade ≥ 2 radiation rectitis (OR = 1.1185 [0.9824–1.2735] by +10 MMPs/µl, p = 0.07)
Conclusion: Our data demonstrate that the levels of circulating PMPs and MMPs are correlated to low and moderate radiation doses rather than to the highest one. These results suggest that these 2 MP subtypes are released after irradiation, though their number reaches a plateau beyond a threshold around the median dose. Furthermore, MMPs appear as predictive of severe rectal complications. These findings suggest that circulating MMPs may be valuable for the prognostic of radiotherapy late complications.

OS23.03

Using machine learning of extracellular vesicle flow cytometry to build predictive fingerprints for prostate cancer diagnosis

Robert Paproski, Deborah Sosnowski, Desmond Pink and John Lewis

University of Alberta, Alberta, Canada

Introduction: Extracellular vesicles (EVs) hold great promise for diagnostics in cancer. Micro-flow cytometry can enumerate and characterise EVs in biological fluids although EV heterogeneity in size, abundance, and marker expression complicates analysis. Our goal was to develop an algorithm capable of predicting clinical outcomes from EVs in bodily fluids.
Methods: Pre-diagnosis plasma samples from 215 men which received prostate biopsies were stained with a variety of markers including prostate-specific membrane antigen (PSMA) and ghrelin and analysed with the Apogee A50 flow cytometer. Informed consent was obtained and the study was approved by the Health Research Ethics Board of Alberta Cancer Committee. Data was loaded into MATLAB, log transformed and particle abundance was determined using multidimensional histograms. Bins per parameter were varied from 2 to 128. Particle abundance within bins was transformed with or without log, z-score, and t-SNE (dimensionality reduction technique) and analysed with 23 different machine learning algorithms to predict aggressive prostate cancer (Gleason 4 + 3 or higher). Fivefold cross-validation was used and repeated 10 times with patient randomisation. Our results were compared with the established Citrus algorithm. We also created synthetic data sets with "shifting" scatter plots to determine if convolutional neural networks could solve this issue.
Results: Using at least 8 bins per parameter generated the best predictive models. The highest AUCs for the LALS-ghrelin, LALS-PSMA, LALS-PSMA-ghrelin and PSMA-ghrelin data sets were 0.56, 0.58, 0.59 and 0.63, respectively. Log and z-score transformation increased AUCs for the LALS-ghrelin and PSMA-ghrelin data sets to 0.61 and 0.69,

respectively. t-SNE transformation increased the PSMA-ghrelin data set AUC to 0.71. Combining the best predictive scores of all 2 parameter data sets provided an AUC of 0.76 which was higher than the 0.64 AUC from Citrus. When analysing shifted synthetic data, convolutional neural networks provided the most accurate results.
Conclusion: We've optimised an advanced algorithm capable of predicting prostate cancer aggressiveness from flow cytometry data. Further integration of deep learning algorithms should improve model performance.

OS23.04

TGFβ3 expression level in extracellular vesicles present in the plasma of patients with head and neck squamous cell carcinoma is a marker for treatment response

Dorival Mendes Rodrigues[1], Soon Sim Tan[2], Sai Kiang Lim[2], Andre Lopes Carvalho[3], N. Gopalakrishna Yier[4] and Andre Luiz Vettore[1]

[1]Universidade Federal de São Paulo, Brazil; [2]A*STAR; [3]Hospital do Câncer de Barretos, Brazil; [4]National Cancer Centre of Singapore, Singapore

Approximately 30% of patients with locally advanced head and neck squamous cell carcinoma (HNSCC) (stage III–IV) treated with cisplatin-based chemoradiotherapy (CRT) have incomplete response to the treatment and there is no biomarker able to prospectively segregate these patients from those who respond to the treatment. It had been shown that TGFβ is a regulator of radiation therapy and promotes heterogeneity and drug resistance in squamous cell carcinoma. Since extracellular vesicles (EVs) are able to carry proteins in the plasma, Cholera toxin B chain (CTB) and Annexin V (AV), which respectively binds GM1 ganglioside and phosphatidylserine, were used to isolate EVs from cell lines and total plasma samples. HNSCC cell line HN120, which were inherently sensitive to cisplatin (WT), and their isogenic cisplatin-resistant (CR) counterpart were evaluated in this study. It was observed that TGFβ3 expression was higher in the CTB-EVs in HN120 CR when compared to HN120 WT. TGFβ3 expression was also examined in plasma samples from 38 HNSCC patients through ELISA sandwich. This assay revealed that TGFβ3 in CTB and AV EVs was significantly lower in the patients presenting complete response to CRT compared to the patients with incomplete response. This study demonstrated that TGFβ3 expression in CTB-EVs or AV-EVs circulating in plasma could be used to determine the better treatment choice, improving the clinical outcome for HNSCC patients, since it would be able to segregate these patients that may respond or not to CRT approach.

OS23.05

EV-associated MMP9 in high grade serous ovarian cancer is preferentially localised to Annexin V-binding EVs

Agnes T. Reiner[1], Sisareuth Tan[2], Stefanie Aust[3], Nina Pecha[3], Mattias Mandorfer[4], Alain R. Brisson[2], Robert Zeillinger[3] and Sai Kiang Lim[5]

[1]BioSensor Technologies, AIT-Austrian Institute of Technology GmbH; [2]UMR-5248 CNRS – University of Bordeaux, France; [3]Molecular Oncology Group, Department of Obstetrics and Gynecology, Medical University of Vienna, Italy; [4]Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University of Vienna, Italy; [5]A*STAR

Among gynaecological cancers high grade serous ovarian cancer (HGSOC) is the most aggressive type and responsible for most deaths. Still there is a lack of biomarkers that are sensitive and specific enough for clinical applications. In order to identify new markers, different extracellular vesicle (EV) types were isolated from ascites of ovarian cancer patients according to their affinities for lipid-binding proteins – annexin V (AV), cholera toxin B chain (CTB) and shiga toxin B chain (STB) – and their protein cargo was analysed. Low signal-to-noise ratios, which are typically a problem when working with biological fluids for biomarker discovery, are circumvented by this approach. Furthermore, contamination by non-EV complexes, like protein aggregates, which are often present in EV isolations, is minimised, because of specific binding to lipids. We've isolated and analysed CTB-binding EVs (CTB-EV), AV-binding EVs (AV-EVs) and STB-binding EVs (STB-EVs) from malignant ascites of patients with ovarian cancer and from non-cancerous portal-hypertensive ascites of patients with cirrhosis. Each of these three EV types has different levels of CD9, CD63, CD71 and ALIX, suggesting that they are unique EV populations. Next we've analysed them for cancer associated proteins and observed that AV-EVs in ascites of patients with HGSOC, but not patients with cirrhosis have higher levels of protein matrix metalloproteinase 9 (MMP9). As MMP9 was not detected in CTB- or STB-EVs, our study validates our approach of using different EV types for optimal biomarker discovery. Furthermore MMP9 in AV-binding EVs could be a HGSOC biomarker with enhanced specificity, because its identification requires detection of two distinct components, i.e. lipid and protein.

OS23.06

Proteome-wide profiling of viable tissue-derived extracellular vesicles for development of early diagnostic biomarkers for colorectal cancer

Satoshi Muraoka[1], Satoshi Nagayama[2] and Koji Ueda[1]

[1]Project for Personalised Cancer Medicine, Cancer Precision Medicine Centre, Japanese Foundation for Cancer Research, Japan; [2]Department of Gastroenterological Surgery, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Japan

Introduction: Early detection of colorectal cancer (CRC) is essential for improvement of prognosis by enabling therapeutic intervention at early stage. Recently, it has been shown extracellular vesicles (EVs) could have potential to be served as attractive biomarker carriers in any body fluids. In this study, to identify novel EV biomarkers for CRC early detection, we performed comprehensive proteome analysis of viable CRC tissue-derived EVs, termed as tissue-exudative EVs (Te-EVs).
Methods: Te-EVs were obtained from serum-free culture media of freshly resected CRC tissues and adjacent normal mucosa using the sequential ultracentrifugation method (n = 20). A quantitative expression profile of Te-EV proteins was acquired by LC/MS. Following protein identification and quantification analysis by MaxQuant software. Four statistically valid biomarker candidate proteins were further evaluated by plasma EV-ELISA assays. Additional clinical and functional assessments were also performed including IHC staining and EV incorporation assays.
Results: Among 6371 identified Te-EV proteins, 616 proteins were significantly overexpressed ($p < 0.05$, fold change >4.0) in EVs from CRC tissues compared to those from paired normal mucosa. We especially focused on four EV membrane proteins as potential biomarkers, TMAM ($p = 3.62 \times 10^{-5}$, fold change = 7.0), STAM ($p = 1.88 \times 10^{-3}$, fold change = 6.0), GAM ($p = 7.46 \times 10^{-3}$, fold change = 4.4) and CSAM (detected only in CRC tissue), since their expression levels in plasma EVs from CRC patients were also significantly higher than those from healthy donors in EV-ELISA assays using independent validation set. IHC staining analysis also demonstrated four EV proteins specifically overexpressed in CRC cells. Interestingly, uptake of $STAM^{++}$ EVs enhanced both proliferation and invasion of recipient cells *in vitro*.
Conclusions:Thus TMAM, STAM, GAM and CSAM on EVs should be potential diagnostic or prognostic biomarkers for CRC, leading to development of precise, non-invasive and low-cost blood liquid biopsy tests in future.

Room: Harbour Ballroom
Symposium Session 24 – EV Functions in Inflammation
Chairs: Saara Laitinen and Takahiro Ochiya 1:30–3:00 p.m.

OS24.01

Extracellular vesicles from adipose-derived mesenchymal stem cells promote autophagy in human osteoarthritic chondrocytes

Miguel Tofiño-Vian[1], Maria Jose Alcaraz[1], Maria Dolores Perez del Caz[2], Miguel Angel Castejon[3] and Isabel Guillen[4]

[1]IDM, University of Valencia; [2]Department of Burn and Plastic Surgery, La Fe University Hospital; [3]Department Orthopaedic Surgery and Traumatology, La Ribera University Hospital; [4]IDM, University of Valencia and Department of Pharmacy, CEU-Cardenal Herrera, Valencia

Introduction: Adipose tissue-derived mesenchymal stem cells (AD-MSC) release extracellular vesicles (EV) both under physiological and pathological conditions. The immunomodulatory and anti-inflammatory properties of AD-MSC have proven to be beneficial in several diseases. Osteoarthritis (OA) is a leading cause of disability in the elderly. Cartilage destruction is mediated by changes in chondrocyte metabolism and the up-regulation of inflammatory or catabolic genes. In OA chondrocytes, the induction of autophagy may be a protective mechanism against stress. We have investigated the effects of microvesicles (MV) and exosomes (EX) from AD-MSC on autophagy, measured as LC3B-positive autophagosome formation, and the production of inflammatory and catabolic mediators in OA chondrocytes stimulated with IL-1β.
Methods: AD-MSC were isolated from fat of patients who undergone abdominoplasty. EV were isolated from AD-MSC conditioned medium by differential centrifugation with size filtration. Tunable resistive pulse sensing was used to evaluate the concentration and size of Ex and Mv. OA chondrocytes were stimulated with IL-1β (10 ng/mL) and treated with MV (3.6×10^7 particles/mL) or EX (7.2×10^7 particles/mL) for 24 h. The levels of oxidised proteins, IL-6, IL-10 and TNFα were measured by ELISA, PGE_2 by RIA, and MMP activity and NO by fluorometry. The expression of collagen II and LC3B was evaluated by confocal microscopy. The data were analysed by ANOVA followed by Dunnett's test.
Results and Conclusion: EV down-regulated the production of inflammatory and degradative mediators induced by IL-1β. Treatment of OA chondrocytes with MV or EX resulted in a significant reduction of MMP activity, oxidative stress, IL-6 and TNFα levels. In addition, they increased the production of the anti-inflammatory cytokine IL-10 and the expression of collagen II. Both types of EV promoted the liberation of LC3B-positive autophagosomes, with a higher effect for MV. Our data indicate that EV exert protective effects on OA chondrocytes and may have potential pharmacological applications to control autophagy, inflammatory processes and extracellular matrix degradation.
Funding: SAF2013-48724-R (MINECO, FEDER) and PROMETEOII/2014/071 (Generalitat Valenciana).

OS24.02

Therapeutic control of systemic inflammation & atherosclerosis with apoe-polarised macrophage exosomes

Robert Raffai, Kang Li and David Wong

University of California San Francisco, CA, USA

Introduction: ApoE expression by myeloid cells has been shown to suppress and even reverse atherosclerosis. We reported that apoE increases microRNA-146a levels to suppress NF-kB activation in monocytes and macrophages and thereby inflammation and atherosclerosis in mice. What is not known is whether macrophage apoE expression modulates microRNA levels in their secreted exosomes to suppress systemic and vascular inflammation via intercellular communication, and whether such exosomes could serve as treatments for atherosclerosis.
Methods: Bone marrow-derived macrophages were cultured from wild-type (WT) mice and ApoE deficient (ApoE-/-) mice. Exosomes were isolated using gradient density ultracentrifugation and assessed by Nano-particle analysis. Global microRNA content in macrophages and their exosomes were assessed by unbiased sequencing. Exosomes were tested for their capacity to alter NF-kB activation in cultured endothelial cells and macrophages. Exosomes were also tested for their capacity to control acute and chronic inflammation *in vivo* by infusing 10E10 particles into WT and ApoE-/- mice every two days for a period of two weeks. Subsequently, WT mice were challenged with sub-lethal LPS and were examined for inflammation in peritoneal macrophages, while levels of Ly6Chi monocytes were detected in the circulation of ApoE-/- mice.
Results: An absence of ApoE expression in macrophages increased exosome secretion and substantially altered their microRNA content. ApoE-/- exosomes enhanced NF-kB activation in cultured endothelial cells and macrophages, and infusions of apoE-/- exosomes enhanced inflammation in peritoneal macrophages of WT mice. In contrast, infusions of WT macrophage exosomes significantly reduced the expression of TNF-alpha and IL-6 in peritoneal macrophages isolated from mice stimulated with LPS. Moreover, WT exosomes caused a two-fold reduction in levels of pro-inflammatory Ly6Chi monocytes in the circulation of ApoE-/- mice.
Conclusions: ApoE expression by macrophages controls the rate of exosome production and their microRNA content to suppress acute and chronic inflammation. Ongoing studies explore whether defined microRNA are responsible for these protective effects and whether such exosomes can be used to suppress atherosclerosis in hyperlipidemic mice.

OS24.03

Apoptotic-cell derived extracellular vesicles are rich in enzymatically-derived active lipid mediators and can modulate immune responses

Ivana Milic[1], Roberta Liccardo[1], Parbata Chauhan[1], Kesley Attridge[1], Helen R. Griffiths[2] and Andrew Devitt[1]

[1]School of Life and Health Sciences, Aston University, Birmingham, United Kingdom; [2]Faculty of Health and Medical Sciences, University of Surrey, Surrey, United Kingdom

Introduction: Apoptosis is a highly orchestrated programme resulting in an active release of apoptotic cell-derived extracellular vesicles (ACdEVs) to communicate their presence and enable efferocytosis. We have shown that ICAM-3 on ACdEVs interacts with macrophages and promotes chemotaxis. Given the molecular complexity of ACdEVs, it is highly likely that other functional mediators (eg proteins, lipids, metabolites) that promote efferocytosis and resolution of inflammation remain unidentified. We aim to address ACdEVs structure-function relationship using a comprehensive multidisciplinary approach.
Methods: Human primary lymphocytes were negatively isolated from peripheral blood. Apoptosis was monitored using annexin V and propidium iodide staining. ACdEVs isolated by centrifugation were characterised using TRPS technology, for EV size and concentration. Presence of EV markers was confirmed by Western blotting. Using a novel downstream multi-omics approach we could simultaneously analyse miRNA, small lipid metabolites and proteins in ACdEVs. Wideband protein and lipid profiling, metabolipidomics were performed using nLC-HR-Q-TOF-MS/MS and LC-QqLIT-MRM, respectively. The functional nature of ACdEVs towards human macrophages was analysed by migration assays.
Results: Apoptosis of primary lymphocytes promotes release of EV that fall within the exosome size range and are positive for exosomal markers (eg TSG101). ACdEVs were shown to promote macrophage chemoattraction. Proteomics of ACdEVs revealed more than 500 proteins, highly enriched in exosomal proteins. A systems biology approach revealed that ACdEVs were enriched in membrane domain proteins involved with cell adhesion,

regulation of immune responses and cell migration. Metabolipidomics revealed an assembly of LOX and COX polyunsaturated fatty acid-derived metabolites, with pro-inflammatory (eg PGD2, PGE2, LtB4), anti-inflammatory and pro resolving properties (eg RvD1, LXA4, LXB4, Mar1).
Summary: Our novel data suggest that ACdEVs may act as a pool of small pro resolving metabolites and their precursors, and adhesion molecules to effect macrophage recruitment, efferocytosis and subsequent immune modulation. Future studies will aim to address the function of a selected panel of protein and metabolite targets.

OS24.04

Histone flow: from nucleus to extracellular vesicles

Rohini Ravindran Nair, Davide Mazza, Alessandra Agresti and Marco Bianchi

University of San Raffaele, Milan, Italy

Introduction: Histones play a central role in DNA packaging and epigenetic regulation. Interestingly, histones are also found as soluble molecules in the blood of sepsis patients (1). Until recently researchers viewed histone content in each and every cell as fixed. Recent reports indicate that histone content decreases in senescent cells. Our group had shown that macrophages treated with LPS decrease their nucleosome content by approximately 20% in 4 h (2). Our aim was to determine the fate of the 20% "missing" histones in macrophages stimulated with LPS.
Methods and Results: First, we evaluated whether stimulated macrophages reorganise their chromatin structure, at a global level. Using quantitative super-resolution microscopy (STORM) we observed that after LPS stimulation of macrophages, nucleosomes clutches (2) reduce both their size and density, suggesting that histones are evicted from chromatin.
Evicted histones can have two possible fates: they can be degraded or secreted out of the cells. To test for histone degradation we collected the cells together with their medium, but we found no difference before and after stimulation. In contrast, histones amount in the medium increased after stimulation. These data imply that histones are not degraded but secreted.
The medium of stimulated macrophages was subjected to ultracentrifugation on an Optiprep density gradient. We found more histones both in extracellular vesicles (EVs) and in the soluble fraction. This result was confirmed using knock-in mice expressing H2B-GFP macrophages which were found to secrete microvesicles containing H2B-GFP.
We excluded that EVs originate from membrane blebbing occurring during apoptosis and necrosis, since there is no significant apoptosis or necrosis in LPS-stimulated macrophages. However, we observed a high level of H3K4 trimethylation in the secreted histones, suggesting that they originate from the nucleus.
We next investigated the localisation of histones in microvesicles: inside or outside the membrane. Biochemical experiments and STROM images indicate that histones are mostly on the outer surface of the vesicles.
Conclusion: Our data show that the nuclear histones can be evicted out of chromatin and be expelled either as soluble protein or microvesicle–associated proteins.

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OS24.05

Chondrocytes derived from mesenchymal stem cells differentiated in the presence of plasma-derived extracellular vesicles from osteoarthritic patients express disease-related genes

Bartijn Pieters, Onno Arntz, Peter van der Kraan and Fons van de Loo

Radboudumc

Introduction: Osteoarthritis (OA) is an age-related musculoskeletal disease and the most common form of arthritis characterised by low grade synovial inflammation and articular cartilage degeneration. Very little is known about the pathophysiological role of extracellular vesicles (EVs) in musculoskeletal diseases, such as OA. In this study, we investigated the effect of plasma EVs from OA patients during chondrogenic differentiation of mesenchymal stem cells (MSCs).
Methods: Plasma-derived extracellular vesicles (pEVs) were isolated from plasma of OA patients and age-matched healthy controls using size-exclusion chromatography. EV containing fractions were characterised according to the ISEV guidelines. Pelleted MSCs were stimulated with TGF-β and BMP-2 to induce chondrogenic differentiation, either in the presence of pEVs isolated from OA patients or healthy controls. After 8 days, RNA was isolated and RT-qPCR was performed to determine the gene expression profiles.
Results: No significant difference was observed in particle concentration, size or protein concentration between OA patients and age-matched controls. In the presence of pEVs from OA patients MSC-derived chondrocytes showed a significant increase in the expression of *MMP-13* (6.1-fold), *RUNX2* (1.9-fold) and *RANKL* (2.3-fold), compared to pEVs from healthy controls. A trend towards higher *ADAMTS5* expression (2.5-fold, $p = 0.0685$) with OA pEVs was also observed. Additionally, we found a significant higher expression of *WISP-1* (24-fold), suggesting activation of the Wnt-pathway. All other proinflammatory genes tested were not significantly different between the two groups.
Summary: A previous study (1) has shown that EVs released from IL-1β stimulated synovial fibroblasts can induce osteoarthritic changes in articular chondrocytes. Here, we show direct evidence that that circulating pEVs from OA patients can enhance OA-related genes in MSC-derived chondrocytes. The expression profile found suggest the presence of Wnt-proteins on pEVs from OA patients, which are known to be involved in cartilage development and we previously have shown that WISP-1 expression is a feature of experimental and human OA (2).

References

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OS24.06

Role of exosomes in the immunopathogenesis of sarcoidosis

Abhay Kumar[1], Rinkee Kumari[2], Deepshi Thakral[3], Samarjit Das[4] and Dipendra K Mitra[5]

[1]Department of TII, All India Institute of Medical Sciences, New Delhi, India; [2]TII, AIIMS; [3]AIIMS; [4]Johns Hopkins University, MD, USA; [5]Department of TII, AIIMS

Introduction: Sarcoidosis is a disease of unknown aetiology and involves formation of non-caseating granuloma in various organs. Exaggerated immune response is the key feature of the disease; however, the exact mechanism of this immune dysregulation is unknown. Few attempts have been made to understand the role of exosomes in immunopathogenesis of sarcoidosis but with limited success. Here, we investigated the impact of BAL fluid-derived exosomes on various immune cells from sarcoidosis patients.
Methods: Exosomes were purified from BAL fluid and plasma by differential centrifugation followed by ultracentrifugation and stored at −80° C. Further, exosomes were analysed for purity and size (by Nanosight and Transmission Electron Microscopy) and stained with fluorescently labelled antibodies (CD81, CD63, CD3, CD14, CD19) for flow cytometric analysis (BD Calibur). Data was analysed using FlowJo software. Their impact on monocytes, regulatory and effector Tcells was determined by flow cytometry.
Results: The monocytes and T-cells derived exosomes were significantly high in BAL fluid of sarcoidosis patients. An increase in the expression of IL-12 and TNFα was observed by autologous monocytes in presence of BAL derived exosomes. Moreover, the frequency of T-cells expressing IFN-γ, TNF-α, IL9 and IL17 were increased, when co-cultured with exosoms from the BAL. In contrast, we observed exosome-dependent decrease in expression of IL10 by Tregs and monocytes. Furthermore, the impact of BAL-derived exosomes was similar but more profound than plasma-derived exosomes on the above immune parameters.
Conclusion: Our preliminary observations indicate an important role of exosomes in inflammatory cytokine production by the monocytes and T cells among sarcoidosis patients. Efforts are underway to decipher the mechanism employed by exosomes for immune-modulation and to identify exosome- based biomarker for the disease.

Room: Metropolitan Ballroom West and Centre
Symposium Session 25 – EV-Mediated Communication in Cancer II
Chairs: Louise Laurent and Dave Carter 3:30–5:15 p.m.

LBO.12

Circulating tumor-associated microparticles in hepatocellular carcinoma and cholangiocarcinoma

Sabine K. Urban[1], Henrike Julich-Haertel[1], Marcin Krawczyk[1], Arnulf Willms[2], Krzysztof Jankowski[3], Waldemar Patkowski[4], Beata Kruk[5], Maciej Krasnodębski[4], Joanna Ligocka[4], Robert Schwab[2], Ines Richardsen[2], Sebastian Schaaf[2], Angelina Klein[2], Sebastian Gehlert[6], Markus Casper[7], Jesus Banales[8], Detlef Schuppan[9], Piotr Milkiewicz[4], Frank Lammert[7], Marek Krawczyk[4], Veronika Lukacs-Kornek[7] and Miroslaw Kornek[1]

[1]Department of Internal Medicine II, Saarland University Medical Center, Saarland University, Homburg, Germany; [2]Department of General, Visceral and Thoracic Surgery, German Armed Forces Central Hospital, Koblenz, Germany; [3]Department of Internal Medicine and Cardiology, Medical University of Warsaw, Warsaw, Poland; [4]Department of General, Transplant and Liver Surgery, Medical University of Warsaw, Warsaw, Poland; [5]Laboratory of Metabolic Liver Diseases, Department of General, Transplant and Liver Surgery, Medical University of Warsaw, Warsaw, Poland; [6]Department of Molecular and Cellular Sport Medicine, Institute of Cardiovascular Research and Sport Medicine, German Sport University Cologne, Cologne, Germany; [7]Department of Medicine II, Saarland University Medical Center, Saarland University, Homburg, Germany; [8]Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, University of the Basque Country (UPV/EHU), CIBERehd, Ikerbasque, San Sebastian, Spain; [9]Institute of Translational Immunology and Research Center for Immune Therapy, University Medical Center, Johannes Gutenberg University, Mainz, Germany

Introduction: Considering the high lethality of liver cancer, new early detection methods are in urgent need to increase patient survival. Here, we aim to improve early diagnosis and therapy monitoring possibilities of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) by applying a minimally-invasive approach involving tumor-associated microparticles (taMPs), large extracellular vesicles.
Methods: TaMPs from patients' sera were isolated by a sequential two-step ultracentrifugation (2,000 and 20,000 g). Their surface antigen composition was analyzed by FACS in order to identify subpopulations associated with the presence of liver tumors or liver cirrhosis, a non-tumor related disease (EV-TRACK ID: EV170006). In total, 172 liver cancer patients (CCA or HCC), 54 cirrhosis patients and 202 control subjects participated in the study. In 27 liver cancer patients a R0 resection was performed.
Results: By identifying AnnexinV$^+$EpCAM$^+$CD147$^+$ taMP populations, HCC and CCA patients could be detected. Moreover, AnnexinV$^+$EpCAM$^+$CD133$^+$ and Annexin V$^+$ EpCAM$^+$ASGPR1$^+$ CD133$^+$ taMPs were capable of discriminating liver disorders (HCC, CCA and cirrhosis) from patients bearing non-liver cancers and from disease-free individuals. Additionally, AnnexinV$^+$EpCAM$^+$ASGPR1$^+$ taMP levels were elevated in liver cancer patients (HCC and CCA combined) by 3.05-fold ($p < 0.0005$) as compared to tumor-free cirrhosis patients. Associated AUROC (0.7), sensitivity (75%) and positive predictive value (78%) implied a potent diagnostic accuracy. During the course of 10 days AnnexinV$^+$EpCAM$^+$ASGPR1$^+$ taMPs decreased from 26.7 (pre-R0 resection) to 7.7 ($p < 0.05$) taMPs per 10^3 Annexin V$^+$ MPs. The smallest detectable liver tumors were 9 mm (HCC) and 11 mm (CCA) in size.
Summary/Conclusion: Our results demonstrate the potential of AnnexinV+EpCAM+ASGPR1+ taMPs as a novel biomarker for HCC and CCA detection. Since their assessment reveals the presence and possibly the extent of these cancers, they feature a minimally-invasive, accurate liquid biopsy screening tool that could considerably improve (early) diagnostics and therapy in patients with primary liver cancer.
Funding: Studies were supported by a German Cancer Foundation grant (111184) to Miroslaw Kornek and by the Alexander von Humboldt Foundation -
SKA 2012 award to Veronika Lukacs-Kornek

LBO.13

Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the pre-metastatic niche

Michael P. Plebanek[1], C. Shad Thaxton[1] and Olga Volpert[2]

[1]Northwestern University, Chicago USA; [2]University of Texas MD Anderson Cancer Center, Houston, USA

Introduction: Metastatic cancers produce exosomes that condition pre-metastatic niches in remote microenvironments to favor metastasis. Here we show that exosomes from poorly metastatic melanoma cells can inhibit metastasis to the lung. These "non-metastatic" exosomes stimulated an innate immune response through the expansion of Ly6C^{low} patrolling monocytes (PMos), which then caused cancer cells clearance at the pre-metastatic niche. This is the first demonstration that pre-metastatic tumors produce exosomes, which elicit a broad range of PMo-reliant innate immune responses, causing cancer cell clearance at the pre-metastatic niche.
Methods: Exosomes were isolated from A375 or B16F10 melanoma cells by differential ultracentrifugation and from patient samples using precipitation followed by CD63/CD81 affinity capture. Mouse models of melanoma were used to show exosomes effects on metastasis, and flow cytometry and immunohistochemistry to determine the immune cell types targeted by exosomes. Additionally, exosomes from the sera of melanoma patients were collected and ELISA was used to determine pigment epithelium-derived factor (PEDF) presence in exosomes.
Results: Our data shows that non-metastatic exosomes drive expansion of PMos as was evident by increased Nr4a1 expression of bone marrow monocytes after treatment with non-metastatic exosomes compared to metastatic exosomes or untreated control cells, as well increased presence of Nr4a1-positive cells in the lungs. Additionally, non-metastatic exosomes contain PEDF as shown, whereas metastatic exosomes are devoid of PEDF. Most importantly, ELISA shows significantly higher amounts of PEDF in the sera exosomes of melanoma patients with a greater than 5-year survival, as opposed to patients with more rapidly progressing disease.
Summary/Conclusion: In this study we discovered that early stage, pre-metastatic melanomas express triggers of immune clearance (PEDF) that are loaded onto the surface of exosomes, activate the innate immune cells PMos and could be developed into potential biomarkers. Lack of PEDF on exosomes is associated with more aggressive disease. Additionally, this study provides an entirely novel mechanism for the increased presence of PMos at the pre-metastatic niche where they recruit NK cells to clear circulating tumor cells from the tumor bearing host.

LBO.14

An extracellular vesicle blood fingerprint distinguishes between patients with indolent and aggressive prostate cancer at diagnosis

John Lewis[1], Robert Paproski[1], Desmond Pink[1], Catalina Vasquez[1], Deborah Sosnowski[1], Bryan Donnelly[2], Adrian Fairey[1], Ron Moore[1], Eric Hyndman[2], Martin Duffy[2] and Jun Kawakami[2]

[1]University of Alberta, Canada; [2]University of Calgary, Canada

Introduction: Prostate cancer is the most commonly diagnosed cancer in men, and early diagnosis is essential to providing curative intervention for those with aggressive disease. Blood PSA levels are currently used to decide whether men will receive an invasive prostate needle biopsy, which provides a diagnosis but comes with significant discomfort and risk of infection. Using a highly sensitive micro-flow cytometry assay and advanced machine learning approaches, we have developed a prostate cancer EV fingerprint that can distinguish between patients with indolent and aggressive prostate cancer at diagnosis using a few drops of

blood. Here we present our initial clinical validation and accuracy of the test in a prospective pre-diagnosis patient cohort.
Methods: Pre-diagnosis plasma samples from 377 Albertan men for whom a prostate biopsy was ordered were analyzed using the Apogee A50 micro-flow cytometer. A panel of biomarkers including prostate-specific membrane antigen (PSMA) and ghrelin was utilized to enumerate specific EV populations from the bulk EVs present in plasma. Using a customized XGBoost machine learning approach with advanced feature selection, a prospective training cohort of 289 patients was utilized to generate an EV fingerprint predictive score (EV-FPS) with a range of 0-100 to distinguish between patients with aggressive prostate cancer (Grade Groups 3-5) from those with indolent prostate cancer (Grade Groups 1 and 2). The EV-FPS was then validated in an independent prospective cohort of 88 patients.
Results: EV-FPS was significantly higher in aggressive (17) vs. indolent (5.8) prostate cancer ($p < 0.0001$). At a sensitivity of 95%, clinical features including PSA provided only 17% specificity for aggressive prostate cancer with an AUC of 0.72. Combining EV-FPS with clinical features at sensitivity 95% increased the specificity for aggressive prostate cancer to 56% with an AUC of 0.83.
Summary/Conclusion: The EV-FPS test significantly increases diagnostic performance for the prediction of aggressive prostate cancer compared to PSA and clinical features alone. Using a score cut-off that achieves 95% sensitivity, men with aggressive prostate cancer can be accurately identified, and up to 56% of men could potentially avoid a biopsy altogether.
Funding: Alberta Cancer Foundation, Prostate Cancer Canada

LBO.15

Molecular subtypes of glioma stem cells as determinants of tumour vesiculome and extracellular vesicle mediated intercellular communication

Cristiana Spinelli[1], Laura Montermini[2], Dong-Sic Choi[3], Brian Meehan[3], Delphine Garnier[4], Shilpa Chennakrishnaiah[5], Esterina D'Asti[2], Ichiro Nakano[6] and Janusz Rak[7]

[1]McGill University, Montreal, Canada; [2]The Research Institute of the McGill University Health Center, Montreal, Canada; [3]The Research Institute of the McGill University Health Center, Montreal, Canada; [4]UMR Inserm 892/CNRS 629 - CRCNA Nantes; [5]Mcgill Center, Montreal, Canada; [6]Department of Neurosurgery, University of Alabama at Birmingham, AL, USA; [7]Montreal Children's Hospital, Research Institute of the McGill University Health Center, Montreal, QC, Canada

Introduction: One of the emerging mechanisms that govern multicellular processes in cancer is the direct exchange of bioactive molecules via intercellular trafficking of extracellular vesicles (EVs). These heterogeneous membrane structures include exosomes, ectosomes, apoptotic bodies and other EV subsets still poorly defined. We have previously uncovered the impact of oncogenic and differentiation processes on biogenesis and function of cancer EVs and wish to extend this to the biology of glioma stem cells (GSC) responsible for the recurrent and incurable nature of aggressive brain tumours know as glioblastoma multiforme (GBM).We propose that biogenesis, properties and biological activity of GBM-related EVs are dictated by oncogenic and epigenetic pathways driving proneural (PN) or mesenchymal (MES) subtypes of GSC populations.
Methods: We isolated and analyzed EVs from cultured GSCs using differential centrifugation nanoparticle tracking (NTA) molecular profiling (sequencing, proteomics, western blot, qRT-PCR) electron microscopy and endothelial bioassays.
Results: We observed that human PN and MES GSC lines, exhibit subtype-specific profiles of EV-related genes (vesiculome) and unique patters of EV formation. Serum-induced differentiation impacted both the GSC phenotypes and EV outputs, including the expression of CD133 (PN) and CD44 (MES) GSC markers, markers of astrocytic (GFAP) or neuronal (TUJ1) lineage commitment. NTA revealed the existence of exosome sized EVs in the GSC conditioned medium which markedly increased in upon differentiation. Proteomic characterization of the EV cargo documented that MES GSCs emit completely different EVs compared to their PN counterparts the latter lacking common exosomal markers. The respective EVs also exhibited different biological activities against endothelial cells, as a function of their subtype and differentiation status.
Summary/Conclusion: With these findings we posit that EVs contribute to and reflect the equilibrium between GBM stem and non-stem cell populations, and they regulate tumour-vascular interactions in a manner influenced by their subtype and differentiation state. Moreover, oncogenic and epigenetic pathways, underlying known trajectories of GBM progression, control EV biogenesis, bioactive cargo, and intercellular interactions.
Funding: MICRTP Studentship 2016

OS25.01

Insights into the mechanisms of neratinib-resistance: investigating a possible role for extracellular vesicles in HER2-overexpressing breast cancer

Michelle C. Lowry[1], Susan Breslin[1], Sinead Toomey[2], Bryan T. Hennessy[2] and Lorraine O'Driscoll[3]

[1]Trinity College Dublin, Ireland; [2]Royal College of Surgeons, London, United Kingdom; [3]School of Pharmacy and Pharmaceutical Sciences and Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland

Introduction: Excluding non-melanoma skin cancer, breast cancer is the most common female cancer and the most common cause of female cancer deaths worldwide. A major issue in the treatment of breast cancer is *de novo* and acquired resistance to therapies. Although neratinib is proving efficacious in HER2+ metastatic breast cancer clinical trials, neratinib-resistance (NR) is an evolving issue. This study aims to determine the mechanisms of NR, discover potential predictive biomarkers and to potentially lead to the discovery of new therapeutic targets in HER2+ breast cancer.
Methods: NR variants of three HER2+ cell lines (EFM19.2A, HCC1954 and SKBR3) were developed by exposing these previously drug-sensitive cells to increasing concentrations of neratinib over a 6 month period. Neratinib IC_{50} for all variants was determined using acid phosphatase assays. Extracellular vesicles (EVs) released from each variant were isolated using ultracentrifugation. To characterise EVs, immunoblotting, nanosight tracking analysis (NTA) and transmission electron microscopy (TEM) were performed. Cellular DNA content was investigated using Sequenom MALDI-TOF MS. Proteomic analysis of cellular and EV content was performed by Olink.
Results: NR variants of the three cell lines were successfully developed, as EFM19.2A-NR, HCC1954-NR and SKBR3-NR. The neratinib IC_{50} for these variants were 6.5-fold, 6.8-fold and 7.4-fold that of their respective parent cell lines. Immunoblotting, NTA and TEM showed successful isolation of EVs from each. DNA Sequenom led to the discovery of 3 SNPs in the HCC1954-parent and HCC1954-NR variants i.e. two SNPs in PIK3CA gene, one SNP in PIK3R1. Of the 181 proteins analysed, some were found to be enriched in EVs compared to cells, others displayed opposite trends. Three proteins (CA9, CSF-1 and TLR3) showed substantial increased quantities in NR variants and their respective EVs, compared to drug-sensitive counterparts.
Conclusions: Further studies are warranted to validate these findings in more cell models, to investigate the functional relevance of CA9, CSF-1 and TLR3 in NR and, subsequently, progress our findings to analysis of specimens from appropriate cohorts of breast cancer patients.
Funding: Irish Cancer Society's support of Breast-Predict and H2020 support of BM1202 ME-HaD.

OS25.02

The role of extracellular vesicle transfer in the heterogeneity of glioblastoma

Jakub Godlewski, E. Antonio Chiocca and Agnieszka Bronisz

Brigham and Women's Hospital, MA, USA, Harvard Medical School, MA, USA

Introduction: Despite the importance of molecular subtype classification of glioblastoma multiforme (GBM), the extent of extracellular vesicle (EV)-driven molecular and phenotypic re-programming remains poorly understood.
Methods: Using intracranial xenografts of patient-derived GBM stem-like cells (GSCs), we identified subpopulations with distinct transcriptomes, displaying either proliferative/nodular or migratory/invasive modes that

were associated with mesenchymal-like or proneural-like subtype, respectively. To reveal complex subpopulation dynamics within the heterogeneous intra-tumoral ecosystem, we characterised protein and microRNA expression and secretion in these phenotypically diverse subpopulations of GSC. Bioinformatic analysis followed by functional EV transfer between GSC *in vitro* and *in vivo* was used to analyse their molecular and phenotype subtype characteristics.
Results The highly heterogeneous profile of microRNAs expression in GBMs was distinguishable into two unsupervised classes that partially overlapped with previously determined molecular subtypes, with both subclasses of GSCs displayed differential cellular and EV microRNA profiles. The analysis of microRNA/target networks provided evidence that EV/microRNAs are modifiers of both the molecular landscape and phenotype, acting via cell type-dependent targeting. Importantly, EV proteome retained the subtype specificity and EV protein signatures were associated with worse outcome. The transfer of EVs between subpopulations of GSCs led to increased tumorigenicity *in vitro* and *in vivo* but did not cause a phenotypic switch, facilitating the formation of inter-dependent tumour organisation.
Conclusions: Our findings demonstrated the existence of previously underappreciated heterogeneity among cancer EVs that contribute to the diverse complexity of the brain tumour ecosystem, indicating that clinical outcome is influenced by the proportion of tumour cells of varying subtypes which by the exchange of EVs can modify molecular landscape and phenotype, acting in tumour anatomic sites-dependent fashion.

OS25.03

Adipose tissue endothelial cell derived microparticles are a potential link between obesity and prostate cancer

Bronson Haynes, Justin Cimring, Ryan Huyck, Lifang Yang, Vanessa L. Correll, Michael McGeagh, Sucharita Dutta, Oliver J. Semmes and Anca D. Dobrian

Eastern Virginia Medical School, VA, USA

Introduction: Obesity increases the risk and aggressiveness of multiple cancers including prostate cancer. Visceral adiposity is a rich source of inflammatory mediators and endothelial cells (EC) from adipose tissue are exposed to such a pro-inflammatory milieu in obesity. We hypothesise that adipose tissue EC exposed to pro-inflammatory cytokines produce microparticles (MP) that contribute to increased tumorigenesis and metastasis of prostate cancer cells via deliveryof a pro-oncogenic mRNA and protein cargo.
Methods: Human adipose tissues EC were treated with TGF-β, IFN-γ and TNF-α and MPs from conditioned media isolated by ultracentrifugation and characterised by particle track analysis and electron microscopy. DIO-labelled MP were incubated with ARCaP-M cells and particle uptake, proliferation and migration were quantified by fluorescence microscopy, BrdU incorporation and invasion assays, respectively. MP proteome was analysed by LC/MS/MS and western blot and mRNA content by RT-PCR.
Results: Cytokine-treated EC produced ~5-fold more MP compared to untreated EC, their uptake by ARCaP-M cells, but not by EC was 2-fold higher, indicating selective tropism for ARCaP-M cells. mRNA for Twist-1 and Snail-1 was significantly increased in MP from cytokine-treated EC. LC/MS/MS revealed protein signatures for Twist-1 and Snail-1, two oncogenes that induce epithelial to mesenchymal transition. In addition, 12-lipoxygenase (ALOX12) mRNA and protein were found in MP and could account for increased MP uptake. MP increased proliferation of ARCaP-M cells and matrigel invasion assay showed a dose-dependent increased migration of MP treated ARCaP cells vs. controls.
Conclusion: MP produced by adipose tissue EC exposed to a pro-inflammatory milieu, such as is the case in obesity contain a pro-oncogenic cargo. ALOX12 and Twist-1 were associated with aggressive prostate tumours in humans. Exposure of human prostate cancer cells to MP produced by EC in pro-inflammatory conditions led to increased proliferation and migration of tumour cells and MP-derived proteins such as Twist-1 and Snail1 may play a key role. This study reveals a yet unrecognised cross-talk between EC-derived MP from human visceral fat and tumour cells and proposes a new link between visceral obesity and prostate cancer.

Room: Metropolitan Ballroom East
Symposium Session 26 – EVs as Epigenetic Regulators
Chairs: Hidetoshi Tahara and TBD 3:30–5:15 p.m.

LBO.16

On-disc Isolation and Analysis of Extracellular Vesicles from Biological Samples

Vijaya Sunkara[1], Hyun-Kyung Woo[2], Juhee Park[3], Tae-Hyeong Kim[3], Chi-Ju Kim[2], Hyun-Il Choi[4], Yoon-Keun Kim[5] and Yoon-Kyoung Cho[6]

[1]Ulsan National Institute of Science and Technology, Ulsan, Republic of Korea; [2]Ulsan National Institute of Science and Technology; [3]Center for Soft & Living Matter, Institute for Basic Science (IBS); [4]Pohang University of Science & Technology, Pohang, Republic of Korea; [5]Pohang University of Science & Technology; Institute of MD Healthcare, Pohang, Republic of Korea; [6]Center for Soft & Living Matter, Institute for Basic Science (IBS); Ulsan National Institute of Science and Technology, Republic of Korea

Introduction: Extracellular vesicles (EVs) are 40 to 1000 nm-sized, cell-derived, membranous vesicles that carry nucleic acids and proteins of the cell of their origin. They are prominent in many body fluids and play diverse roles in intercellular communications. Despite of the increasing interest as potential biomarkers, current methods of their isolation and analysis suffer from the limitations such as requirement of expensive equipment, long processing time or low yield and purity of the vesicles. To address some of the issues, we have demonstrated the use of an Exodisc for isolation and subsequent analysis of the EVs from culture media and urine. Currently, the ability of the Exodisc for isolation of EVs from plasma and other biological fluids are being studied.
Methods: The channels and chambers were fabricated on a polycarbonate (PC) disc by CNC micromachining. A pushpin valve was integrated to control the flow of the fluid. The device consists of two nano-porous membranes with pore sizes of 600 nm (track-etched PC membrane) and 20 nm (AAO membrane). First, the debris was sediment and then solution was passed through the two filters sequentially, by spinning the disc at 3000 rpm. The EVs > 600 nm gets trapped on filter I and those between 20 to 600 nm on filter II. Finally, EVs on filter II were washed with PBS and either analyzed by ELISA on the disc or transferred to a collection chamber for retrieval.
Results: In the Exodisc, starting with raw sample, whole procedure from sample preparation to EVs detection is achieved within one hour. The data shows that the on-disc filtration isolates about four times higher EVs, and analysis of the EV mRNA also shows >100-fold higher concentration of mRNA compared to UC. In addition, the device could able to differentiate the urinary EVs from bladder cancer patients to that of healthy donors, by performing on-disc ELISA utilizing their CD9 and CD81 expressions.
Summary/Conclusion: The Exodisc provides rapid isolation, higher recovery as well as high-sensitive protein detection of EVs compared to conventional methods. The EVs enriched on 20 nm filter can either be retrieved as pristine and intact EVs for conventional analyses or detected on the same device by using specific detection antibodies, promising its potential utility in the EV field.
Funding: HI12C1845, IBS-R020-D1, and SRC (2010-0028684) funded by the Korean Government.

LBO.17

High resolution size exclusion chromatography allows detailed study of exosome heterogeneity

Eduard Willms[1], Pieter Vader[2], Matthew J. Wood[3], Simonides Immanuel van de Wakker[1], Olivier Gerrit de Jong[1], Imre Mäger[3], Samir El Andaloussi[4] and Carlos Cabañas[5]

[1]Professor Matthew Wood Lab; Department of Physiology, Anatomy and Genetics; University of Oxford, United Kingdom; [2]University Medical Center Utrecht, The Netherlands; [3]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom; [4]University of Oxford, United Kingdom; [5]Complutense University of Madrid; Department of Microbiology; Spain

Introduction: Cells release membrane enclosed vesicles termed extracellular vesicles (EVs) that function as mediators of intercellular communication. Exosomes, EVs released upon fusion of the multi-vesicular body and cell membrane, are thought to represent a population of EVs with homogenous biophysical and functional characteristics. However, increasing evidence highlights that exosomes are a heterogeneous population of EVs (Willms et al. 2016, Collino et al. 2017). Here, we employed a two-step size exclusion chromatography approach to identify multiple exosome subpopulations with distinct composition and function.
Methods: Exosomes were isolated from cell culture supernatants using size exclusion chromatography (SEC). Subsequently, exosomes were subjected to fractionation by high resolution size exclusion chromatography (HR-SEC). Dot blot analysis was performed on individual HR-SEC fractions to determine expression of common exosomal markers. Based on expression patterns of these markers, individual HR-SEC fractions were pooled to obtain exosome subpopulations. Western blot analysis was performed to study the composition of the subpopulations, and particle size was determined using nanoparticle tracking analysis. Functional effects on recipient cells were studied using proliferation and migration assays.
Results: Fractionation of isolated exosomes using HR-SEC revealed that exosomes represent a heterogeneous population of EVs. Dot blot analysis on individual HR-SEC fractions demonstrated a distinct distribution of common exosome markers. Exosome subpopulations were identified based on differential expression of common exosomal proteins and previously identified exosome subpopulation markers (Willms et al. 2016). Exposure of recipient cells to subpopulations resulted in differential functional effects.
Conclusion: In conclusion, we demonstrate that exosomes represent a heterogeneous EV population. HR-SEC allows for in depth-study of exosome heterogeneity and identification of exosome subpopulations with distinct biophysical and functional characteristics. Increased understanding of exosome heterogeneity will allow for more detailed study of exosome biology and will facilitate biomarker discovery as well as highly specific engineering of exosomes.

LBO.18

EVQuant: Combined quantification and phenotypic analysis of individual extracellular vesicles in experimental and clinical samples

Thomas Hartjes[1], Diederick Duijvesz[2], Roy van der Meel[3], Mirella Vredenbregt[2], Matthijs Bekkers[2], Raymond M. Schiffelers[4], Adriaan Houtsmuller[1], Guido Jenster[2] and Martin van Royen[1]

[1]Department of Pathology/Erasmus Optical Imaging Centre, Erasmus Medical Center, Rotterdam, The Netherlands; [2]Department of Urology, Erasmus Medical Center, Rotterdam, The Netherlands; [3]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada/Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands; [4]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands

Introduction: Extracellular vesicles (EVs) are an important biomarker source for a range of diseases. Proteins on the surface of secreted organ- or disease-specific EVs in body fluids could be used for detection or monitoring disease. Although various methods exist to quantify EVs, EV quantification in clinical samples remains challenging and more importantly, current approaches are often unable to identify EV subpopulations. Here we provide a microscopy based assay (EVQuant) to both quantify and phenotype individual EVs without the need for EV isolation/purification.

Methods: In short, EVs are labelled using a fluorescent membrane dye and/or immunofluorescent antibodies. To enable detection of low intensity signals, EVs are immobilized in a transparent medium and detected using confocal microscopy or a high-throughput imaging system. Fluorescent EV signals are quantified using open source software. Liposomes were used to identify the size limitation for detection. EVs from 10 different cell lines were quantified and phenotypically analysed by combining general membrane labelling and specific labelling of the EV markers CD9 and CD63 using fluorescent antibodies. The CD9 and CD63 distribution was compared to CD9 and CD63 time-resolved fluorescence immunoassay (TR-FIA) analysis of the same samples.
Results: Quantification of liposomes showed EVQuant was able to detect EVs down to 50nm in size. Multicolor imaging of individual EVs allowed the detection of EV sub-populations and showed a large variation in the presence of the general markers CD9 and CD63 on EVs between cell lines. Concentrations of CD9 or CD63 positive EVs were compared to presence of CD9 or CD63 quantified by TR-FIA and showed no direct correlation which could be partially explained by differences in the average number CD9 and CD63 molecules present on EVs between cell lines.
Summary/Conclusion: EVQuant is a rapid, robust, widely accessible assay with the following benefits; the ability to detect vesicles down to 50 nm in size, no EV isolation/purification needed, and the possibility to perform multicolor imaging. The ability to detect EV sub-populations based on specific biomarkers and the possibility to analyse EV samples in high-throughput, makes EVQuant a suitable candidate for implementation in a clinical setting.
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LBO.19

High sensitivity, quantitative epitope analysis of plasma EVs by flow cytometry

Aizea Morales-Kastresana[1], Xiaomei Yan[2], Shaobin Zhu[3], Katherine McKinnon[4], William Telford[5], Veena Kapoor[5], Jay A. Berzofsky[4] and Jennifer C. Jones[4]

[1]National Cancer Institute, National Institutes of Health; [2]Xiamen University; [3]nanoFCM, Inc., Fujian, Chyina; [4]National Cancer Institute, Vaccine Branch; [5]National Institutes of Health

Introduction: Most conventional flow cytometers are unable to resolve individual 30 – 200 nm extracellular vesicles (EVs), which are likely to carry fewer than 100 copies of any specific epitope. Typically, these EVs are not only too small, but also too dimly-labeled to be individually classified as "positive." Although the limits of scattered light detection are well described, there are no comprehensive reports that delineate the molecular limits of resolution of modern flow cytometers, in terms of how many epitopes or fluorophores are required for detection.
Methods: EVs were isolated size exclusion chromatography and ultrafiltration. We determined Mean Equivalent Soluble Fluorophore (MESF) limits of several instruments, and compared these values with a next generation high sensitivity, avalanche photodiode (APD)-enabled flow cytometer (nanoFCM). Next, we assayed the plasma EV expression of more than 300 epitopes, using PE-conjugated monoclonal antibodies against human cell surface markers and isotype controls. Results: Most conventional flow cytometers cannot detect signals from fewer than 100-1000 fluorophores. Therefore, many EVs that carry low or intermediate numbers of any specific surface molecule will be too dim to be detected by fluorescence with those instruments. However, the nanoFCM demonstrated 10-100-fold higher sensitivity, and a commensurate ability to detect epitope-positive EVs that are too dim to be detected with most available flow- or image- cytometers. Not surprisingly, we found that unbound labels must be removed prior to running samples on the nanoFCM, to achieve maximal sensitivity.
Summary/Conclusion: Due to the diversity of EV sources and biological effects, a longstanding goal of the EV research community is to define relevant EV repertoires and their associated surface epitopes. This is the first comprehensive, quantitative comparison of limits of detection for several flow cytometers, with respect to the detection of fluorescently labeled surface molecules. The nanoFCM enables detection of low- to intermediate-levels of EV surface markers, and our results provide a benchmark profile for high-sensitivity plasma EV epitope detection for more than 300 cell surface epitopes.
Funding: National Institutes of Health, NCI-CCR Vaccine Branch, Radiation Oncology Branch, & Assistant Clinical Investigator Program.

OS26.01

Extracellular vesicle and miRNA profiling of the primate cervicovaginal compartment reveal possible anti-HIV defences

Zezhou Zhao, Dillon Muth, Kathleen Mulka, Bonita Powell, Kelly Metcalf Pate, Zhaohao Liao and Kenneth Witwer

The Johns Hopkins University School of Medicine, MD, USA

Introduction: We previously observed changes in overall concentration of extracellular particles including extracellular vesicles (EVs) recovered from cervicovaginal lavage (CVL) and other fluids in endometriosis and in HIV/SIV infection. Here, we further characterise EVs released during the menstrual cycle and retroviral infection and develop a reference profile of small RNAs of the primate cervicovaginal compartment.
Methods: CVL of rhesus macaques, previously collected over the course of five weeks, were subjected to differential centrifugation to enrich EVs. Characterisation was performed by NTA and WB for EV markers. A medium-throughput qPCR platform was used to profile miRNAs in CVL, swabbed secretions, enriched EVs and biopsy samples, and results were validated by individual qPCR. miRNA function in relation to HIV infection was assessed in monocyte-derived macrophages using HIV-1 BaL or green fluorescent protein-encoding HIV.
Results: EVs with standard EV markers were successfully enriched from CVL. However, miRNAs were present predominantly in EV-depleted fractions of CVL, not in EV-enriched centrifuge pellets. The most abundant miRNAs across fractions were miRs-223, -203, -24, -150, -146a, -21, -222, -92a, -17 and -106a, with only a few miRNAs enriched in EVs. Surprisingly, few miRNAs profile changes were observed during the menstrual cycle or during SIV infection, in either CVL or EVs. However, several abundant CVL miRNAs, including miR-223, may be generally protective against retroviral infection, as suggested by *in vitro* infection assays.
Conclusions: We have established a miRNA profile of CVL fractions and probed the overlap of CVL and EV miRNAs with those found in secretions and epithelial tissues. Although menstrual cycle and SIV infection have only minor effects on CVL miRNA profiles, CVL miRNAs may contribute to antiviral defences. Additional studies are underway to elucidate the role of EV small RNAs in protecting against retroviral reproductive tract infection.

OS26.02

Inflammatory glia alter synapse stability via the transfer of extracellular vesicle-associated miRNAs

Ilaria Prada[1], Elena Turola[2], Martina Gabrielli[3], Giulia D'Arrigo[4], Alessia Iorio[1], Giuseppe Legname[4], Dan Cojoc[5], Marta Fumagalli[1], Francesca Peruzzi[6] and Claudia Verderio[3]

[1]Università degli Studi di Milano, Milan, Italy; [2]Gastroenterology Unit, Department of Internal Medicine, University of Modena and Reggio Emilia, Italy; [3]CNR-IN Neuroscience Institute, Department of Medicine, Milan, Italy; [4]Department of Neuroscience, Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste, Italy; [5]CNR-IOM Institute of materials,Trieste, Italy; [6]Department of Medicine, Scott Cancer Center, New Orleans, LA, USA

Introduction: Beyond the classical secretory mechanism through which glial cells influence brain activity, astrocytes and microglia, release circular membrane fragments, known as extracellular vesicles (EVs). EVs contain several components of the donor cell (RNAs, proteins, lipids) and can transfer their cargo to recipient cells. Our aim is to investigate whether glia can regulate neuron gene-expression through the secretion of EVs.
Methods: Rat primary cell cultures, EV isolation, RealTime-PCR, *Renilla*/Luciferase-based assay, transfection, immunocytochemistry, western blot, optical manipulation and live imaging.
Results: Using miRNA real-time-PCR panels, we identified a set of miRNAs differentially expressed in EVs produced by pro-inflammatory compared to pro-regenerative microglia. Among them, we found miR-146a, a known miRNA involved in inflammatory responses, which is also altered in brain disorders and targets neuron-specific genes. To investigate possible glia-to-neuron shuttling of miR-146a, we performed a Renilla/Luciferase-based assay transfecting rat hippocampal neurons with a miR-146a-specific sensor, and exposing them

to EVs for 24 h. Neuron exposure to glial EVs caused an increase in neuronal miR-146a levels, with a consequent decrease in protein expression of validated miR-146a targets, such as the synaptic vesicle protein synaptotagmin 1 and the postsynaptic adhesion protein neuroligin 1. Morover, this effect resulted in decreased dendritic spine density and reduced number of excitatory synapses in target neurons. Donor glia transfection with miR-146a inhibitor or blockade of phosphatidyl-serine residues on glial EVs, a determinant for EV binding on neurons, prevented the up-regulation of miR-146a and the consequent down-regulation of its downstream targets in neurons. Additionally, by visualising single EV-neuron contacts driven by optical manipulation we observed that EVs form stable interactions with neurons, ruling out the possibility that EVs undergo rapid internalisation or full fusion.
Conclusions: Our data indicate that reactive glia may influence neuronal activity by regulating the translation of crucial components of the synapse through secretion of miR146a-storing EVs.

OS26.03

Stressing out the neighbours: stressed exosomes ("sexosomes"?) passage stress phenotypes to recipient cells

Jasmina Redzic[1], Tom Anchordoquy[1] and Michael W. Graner[2]

[1]University of Colorado Denver, Anschutz Medical Campus, Skaggs School of Pharmacy, CO, USA; [2]University of Colorado Denver, Anschutz Medical Campus, Department of Neurosurgery, CO, USA

Cancer cells undergo a number of stresses, many of them self-inflicted, but often do not appear to suffer the consequences of those stresses. In some cases, the stress responses may actually prove beneficial to the tumour cells, providing them with potent resilience to their less-than-hospitable environments. One consistent tumour stress is the unfolded protein response (UPR), an endoplasmic reticulum-based stress-management system with sensors, transducers, and effectors that result in a transcriptional and translational landscape rearrangement leading to resolution of the stress, or cellular apoptosis. However, tumours may incorporate the UPR into their stress portfolio to survive or even thrive amidst their environmental insults. We propose that exosomes from stressed cells (stressed exosomes, or "sexosomes") are able to induce stress response phenotypes in recipient, unstressed cells, thus enabling stress responses without having to experience the actual stress. Our analysis in this report goes to the molecular level, monitoring proteome changes in glioma cells when those cells are exposed to exosomes released from UPR-stressed cells. We find high overlap in the proteomes of stressed cells and unstressed cells that receive "sexosomes", suggesting that tumours may unify their overall stress responses despite their inherent heterogeneity. The implications for general tumour biology, and in particular, therapeutic resistance, are highlighted.

Room: Harbour Ballroom
Symposium Session 27 – EVs in Cancer Progression and Therapy
Chairs: Andries Zijlstra and Peter Quesenberry 3:30–5:15 p.m.

LBO.22

Ghost nanovesicles for targeted delivery of chemotherapeutics

Gyeongyun Go[1], Changjin Lee[2], Hyun Taek Park[1], Nhung Thi Hong. Dinh[1], Dong-Sic Choi[3], Su Chul Jang[4] and Yong Song Gho[1]

[1]POSTECH; [2]Postech; [3]The Research Institute of the McGill University Health Centre, Montreal, Canada; [4]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden

Introduction: Extracellular vesicles are endogenous nanocarriers that can deliver cellular molecules between cells and they are recognized as alternative targeted drug delivery systems. However, extracellular vesicles are produced in low quantities and the vesicles are filled with cellular molecules that may interrupt efficient drug loading. Here, we developed ghost nanovesicles in which molecules entrapped by cell membrane are released by opening cell membrane under high pH and the membrane are resealed in neutral condition.
Methodsr: Cell membrane sheets of human monocytic U937 cells were isolated by sonication and ultracentrifugation of the cells lysed in sodium carbonate solution. The drug-loaded ghost nanovesicles were generated by further sonicating the membrane sheets in the presence of the drug under neutral pH. Characteristics of the ghost nanovesicles in terms of size, topology, and protein, nucleic acid components were analyzed. Specific uptake and drug delivery of ghost nanovesicles were examined on TNF-α stimulated human umbilical vein endothelial cells (HUVEC) *in vitro*.
Results: Electron microscopy and dynamic light scattering analyses revealed that the ghost nanovesicles were intact membrane vesicles with 120 nm average size. Topology analysis showed that the ghost nanovesicles preserve the original membrane topology. Western blot and qPCR results showed that the ghost nanovesicles are de-enriched with cytosolic proteins, nucleic acids while the nanovesicles enriched with membrane proteins for targeted delivery. The ghost nanovesicles retained natural targeting characteristic of source cells and showed targeted drug delivery on TNF-α stimulated HUVEC.
Summary/Conclusion: These results suggest that the ghost nanovesicles can serve as a novel drug delivery system to achieve effective loading and specific delivery of chemotherapeutics to target cells.

LBO.21

Duotype-specific peptides as tool for tumor progression monitoring via tumor-derived exosomes targeting

Enrico Iaccino[1], Selena Mimmi[1], Ileana Quinto[1] and Giuseppe Scala[2]

[1]Department of Experimental and Clinical Medicine University; [2]Department of Experimental and Clinical Medicine University "Magna Graecia" of Catanzaro

Introduction: In contrast to invasive tissue biopsy, exosomes are effective biomarkers in the diversified diagnosis of personalized medicine. Although several methods have been developed to purify exosomes, none of these can clearly discriminate between normal and tumor-derived exosomes (TDEs) and to completely avoid contaminations by other shed membranes. Recently we reported that small peptide ligands (Id-peptides) targeting the immunoglobulin B-cell receptor are a unique tool to target both transformed and clonogenic precursors of B-cell malignancies. As shown in recently published works, MM released exosomes constitutively express the Ig-BCR making MM secreted exosomes a reliable target for id-peptides binding.
Methods: The monoclonal immunoglobulin (Ig-BCR) from the 5T33MM cell line was purified by Protein G affinity chromatography and utilized as a bait to isolate binders of their antigen-binding site using a C7C phage-displayed peptide library fused to the M13 minor coat protein. Synthetic peptides corresponding to the peptide insert of phage clones were assayed for their antigenic properties out from the phage context, while their specific binding to the target cells was assayed by flow cytometer using fluorescein isothiocyanate (FITC)-conjugated Id-peptides.
Results: The Id-peptides capability to specifically target 5T33MM-derived exosomes was confirmed both *in vitro* and ex vivo. Before these validations, exosomes were first purified using standard procedures and then characterized by scanning electron microscope, nanosizer and Western blotting analysis. Streptavidin magnetic beads were first decorated with biotinylated anti-CD63, incubated with the serum-derived exosomes and then analyzed for FITC-conjugated Id-peptides specificity by flow cytometry. The ex vivo experiments were conducted using the C57BL/KaLwRij strain and their survival rate was evaluated. Blood samples from 5T33MM injected mice were collected every seven days post cells inoculation and serum exosomes were evaluated for Id-peptides targeting in comparison with paraprotein concentration.
Summary/Conclusion: According to the above-mentioned results, this work provides an innovative approach to the early detection and clinical evaluation of the selected disease opening of new horizons for a more comprehensive knowledge of exosomes biological functions.

LBO.20

Mesenchymal stem cell-derived exosomes promote neurologic recovery in experimental autoimmune encephalomyelitis model of multiple sclerosis

Milad Riazifar[1], Egest Pone[1], Ashish Yeri[2], Cecilia Lässer[3], Ganesh Shelke[4], Elizabeth Hutchins[2], Erika Calle[1], Ashley Hamamoto[1], Rossella Crescitelli[4], Wenbin Liao[1], Victor Pham[1], Aude Segaliny[1], Yanan Yin[5], Craig Walsh[6], Kendall Jensen[7], Jan Lotvall[8] and Weian Zhao[1]

[1]Department of Pharmaceutical Sciences, University of California, Irvine, California 92697; [2]Neurogenomics Division, Translational Genomics Research Institute, Phoenix, AZ, USA; [3]Krefting Research Centre, University of Gothenburg, Sweden; [4]Krefting Research Centre, Institute of Medicine, University of Gothenburg; [5]The Krefting Research Centre, Institute of Medicine, The Sahlgrenska Academy, Göteborg University, Sweden; [6]Department of Molecular Biology and Biochemistry, Sue and Bill Gross Stem Cell Center, Multiple Sclerosis Research Center, University of California, Irvine, CA, USA; [7]Neurogenomics, Translational Genomics Research Institute; [8]Codiak BioSciences

Introduction: Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) in which T cells attack the CNS, resulting in demyelination, neuronal injury and death, leading to clinical neurological deficits. Preclinical studies have revealed immunosuppressive properties of mesenchymal stem cell (MSC) to treat MS. However, lung entrapment, maldifferentiation, phenotype change and potentially tumor formation are current challenges for stem cell therapy. The much smaller size of MSC-derived exosomes (Exo) allow reduced lung entrapment, achieve superior biocompatibility, and are more stable and expose fewer risks, which together make them an attractive alternative to MSC.
Methods: Exo were obtained from MSC cultures and were injected I.V. in EAE model of MS.
Results: Here, using experimental autoimmune encephalomyelitis (EAE) as MS mouse model (n=30), we show that systemic injection of MSC-Exo (150 ug) results in sustained recovery and improved motor function ($p< 0.01$). This recovery is associated reduced in neuroinflammation and increased re-myelination ($p< 0.05$). Biodistribution experiments show that Exo were mostly found in liver and sple

LBO.23

Identification of cancer-derived large oncosomes in urine samples of prostate cancer patients

Tatyana Vagner[1], Dolores Di Vizio[2], Valentina R Minciacchi[3], Mandana Zandian[4], Edwin M. Posadas[2] and Andries Zijlstra[5]

[1]Department of Surgery, Cedars-Sinai Medical Center, CA, USA; [2]Cedars Sinai Medical Center, CA, USA; [3]Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt, Germany; [4]Division of Cancer Biology and Therapeutics, Departments of Surgery, Biomedical Sciences and Pathology and Laboratory Medicine, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, US; [5]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA

Introduction: Large oncosomes (LO) are atypically large (1-10μm) cancer-derived extracellular vesicles (EVs), selectively shed by cancer cells with highly migratory properties. LO are visible by microscopy in tumor tissues as they blebb off the plasma membrane of cancer cells during "amoeboid" migration. LO are emerging as a promising source of circulating biomarkers because they contain several types of biomolecules, which represent molecular signatures of their cells of origin. LO have been identified in tumor tissue and in plasma of prostate cancer patients, but their presence in urine has never been examined. The use of urine in clinical tests has several advantages compared with other biofluids, such as blood, because urine is collected non-invasively and the procedure is fast and cost-efficient. The aim of this study was to identify molecular markers that would allow detection of LO in patient urine samples.
Methods: Tunable Resistive Pulse Sensing (TRPS), differential and density gradient ultracentrifugation; western blot; mass spectrometry, flow cytometry.
Results: From mass spectrometry analysis, we found that CK18 was highly abundant in large EVs, while the tetraspanin CD81 was expressed at negligible levels. We validated these findings *in vit*ro using EVs isolated from prostate cancer cell line PC3 and showed that CK18 was enriched in large EVs. In contrast, CD81 was over-represented in nano-sized EVs. Similar results were obtained by spiking in PC3 EVs into the urine of normal subjects, which allowed us to generate urine benchmark LO and exosome samples. TRPS and flow cytometry both identified large EVs in urine of prostate cancer patients. These EVs could also be identified with molecular markers of LO and exosomes, CK18 and CD81 respectively, suggesting that the large EVs detected in urine represent LO. Finally, to determine if urine LO can provide valid prostate cancer biomarkers, we correlated enumeration and molecular cargo of LO from urine and plasma of the same patients.
Summary/Conclusion: Our results indicate that tumor-derived LO can be identified in urine of prostate cancer patients, thus providing an alternative, non-invasive access to circulating diagnostic/prognostic molecular signatures of cancer cells.
Funding: National Institutes of Health NIH UCLA SPORE in Prostate Cancer award P50 CA092131; DoD PCRP Award PC150836 (to DDV).

OS27.01

Effect of stem cell-derived extracellular vesicles on tumour angiogenesis

Benedetta Bussolati[1], Tatiana Lopatina[2], Cristina Grange[2], Marta Tapparo[2], Adriana Pitino[3], Ciro Tetta[4] and Giovanni Camussi[2]

[1]Department of Molecular Biotechnology and Health Sciences, University of Torino, Italy; [2]Department of Medical Sciences, University of Torino, Italy; [3]Molecular Biotechnology Centre; [4]Unicyte AG

Introduction: Endothelial cells present in tumour are characterised by increased angiogenic properties that may contribute to tumour development, growth and metastatisation. Extracellular vesicles from human liver stem cells (HLSC-EVs) and mesenchymal stem cells (EV-MSCs) were reported to display an anti-tumour effect in a number of experimental models. However, the effect of these EVs on tumour angiogenesis has not been studied yet.
Methods: We here evaluated the anti-angiogenic potential of stem cell EVs on tumour-derived endothelial cells (TECs) obtained from renal carcinomas. In particular, we tested the effect on tube formation, proliferation, invasion, motility and apoptosis *in vitro*. *In vivo*, we tested the anti-angiogenic effect SC-EVs on a model of tumour angiogenesis obtained by TEC subcutaneous implantation in SCID mice. Biodistribution of intravenously injected EVs was also studied. The molecular effect of EVs on TECs was investigated using gene array analysis.
Results: HLSC-EVs inhibited the angiogenic potential of TEC *in vitro* and reduced TEC survival and organisation into vascularised structures *in vivo*. No effect was observed for MSC-EVs. Injected EVs were localised into the vascular structures formed by EVs *in vivo* and showed an increased presence in respect to adjacent normal skin vessels. Finally, microarray analysis performed on HLSC-EV-treated TECs identified down-regulation of several pro-angiogenic genes, including HIF-1, VEGF, S1PR1, Integrin β3 and TGF-β, as compared to untreated cells.
Conclusion: HLSC-EVs but not MSC-EVs displayed an anti-angiogenic effect on TECs and inhibited pathways involved in tumour angiogenesis that may contribute to the anti-tumour effect of HLSC-EVs.

OS27.02

RAB7 and prion protein modulate the secretion of extracellular vesicles and show a prognostic value in head and neck squamous cell carcinoma

Fernanda Giudice, Bruna Rodrigues, Tonielle Lacerda, Antuani Baptistella, Marcos Salles, Luiz Paulo Kowalski and Vilma Regina Martins

A.C. Camargo Cancer Center, Sao Paulo, Brazil

Background: Studies have pointed that Rabs, in particular Rab7, modulate extracellular vesicles (EVs) secretion. In addition, we have recently demonstrated that Prion protein (PrP^C) induces exosome secretion through activation of caveolin and impairment of autophagy. The head and neck squamous cell carcinoma (HNSCC) is one of the six most common malignancies worldwide with a high local invasion and metastasis. The characterisation of biomarkers associated with HNSCC progression is very important since it may dictate the prognosis and indicate its best treatment.
Material and Methods: EVs secreted from HNSCC cell lines were isolated by ultracentrifugation and quantified. Rab7 and PrP^C were overexpressed or knockdown in these cells and the secretion of EVs and the cellular invasion were quantified. Rab7 and PrP^C expression were also evaluated in 223 HNSCC specimens in tissue microarrays using immunohistochemistry (approved by the Institutional Ethics Committee-1791/13) and compared to clinical and pathological data. Experiments were compared using one-way ANOVA and patient data and immunohistochemistry results were analysed using chi-square, Kaplan Meier and log-rank tests.
Results: In cell lines RAB7 expression decreases the secretion of EVs and cellular invasion while PrP^C expression increases the secretion of EVs and cellular invasion. Patients with HNSCC presenting higher levels of RAB7 or lower levels of PrP^C show a better prognosis with lower cancer recurrence or cancer-related death during 5 years of follow-up than those expressing lower levels of RAB 7 or higher levels of PrP^C.
Conclusion: Our data suggest the importance of RAb7 and PrP^C expression as prognostic markers for HNSCC. The correlation of EVs secretion from HNSCC and tumour recurrence and disease free survival is under evaluation.

OS27.03

Epigenetic dysregulation of hematopoietic stem cell function by extracellular vesicle (ev) trafficking in the leukaemia microenvironment

Sherif Abdelhamed[1], Noah Hornick[2], Ben Doron[1], Young me Yoon[1] and Peter Kurre[1]

[1]Pediatric Cancer Biology, Knight Cancer Institute, Oregon Health and Science University, OR, USA; [2]Oregon Health and Science University, OR, USA

Acute myeloid leukaemia (AML) is a heterogeneous blood cancer that is associated with the progressive loss of normal bone marrow function. We recently reported that extracellular vesicles (EVs) released from AML cells, but not healthy controls, directly suppress hematopoietic progenitor function via miR155 and miR150 targeting of cMyb, a highly regulated transcription factor expressed in differentiating progenitors. To determine the impact on long-term hematopoietic stem cells (LT-HSC) we used a combination of *in vivo* AML xenografting and intrafemoral EV injections in immunodeficient mice. We observed a significant increase in the frequency of the LT-HSC with gains in quiescence (G0) an increase in DNA damage, evidenced by gH2AX foci and transcriptional upregulation of *Rad51* and *p21* gene expression. Along with an increase in phosphorylation of the tumour suppressor p53, these changes were reminiscent of changes seen during HSC ageing. We reasoned that a mechanism other than the progenitor specific transcription factor c-Myb would have to account for the deregulation of LT-HSC. In a miRNA survey we identified miR-1246 as highly abundant in AML-EV and used our recently reported RISCtrap discovery pipeline and identified a set of targets enriched for negative cell cycle regulators and epigenetic modifiers, Dnmt1 and Hells among them. We propose that AML-EV miRNA lead to epigenetic changes and studies to identify differentially methylated genomic regions that account for the coincident accumulation of DNA damage in quiescent LT-HSC are underway. The epigenetic regulation of hematopoietic stem cells by EV miRNA presents a novel paradigm and the identification of differentially methylated targets may help ameliorate morbidity and mortality from the suppression of hematopoiesis in the AML patients.

OS27.04

Acute myeloid leukaemia transforms the bone marrow niche into a leukaemia-permissive microenvironment through exosome secretion

Bijender Kumar[1], Mayra Garcia[1], Lihong Weng[1], Xiaoman Jung[1], Jodi Murakami[1], Xingbin Hu[2], Tinisha Mcdonald[1], Allen Lin[1], Ashish Kumar[3], David DiGuisto[4], Vinod Pullarkat[5], Susanta Hui[5], Nadia Carlesso[1], Ya-Huei Kuo[1], Ravi Bhatia[6], Guido Marcucci[1] and Ching-Cheng Chen[1]

[1]Beckman Research Institute of City of Hope, CA, USA; [2]Research Associate; [3]Cincinnati Childrens's Hospital, OH, USA; [4]Stanford Hospital and Clinics, CA, USA; [5]City of Hope National Medical Center, CA, USA; [6]University of Alabama Birmingham, AL, USA

Little is known about how leukaemia cells alter the bone marrow (BM) niche to facilitate their own growth and evade chemotherapy. Here, we provide evidence that acute myeloid leukaemia (AML) blasts remodel the BM niche into a leukaemia-growth-permissive and normal-hematopoiesis-suppressive microenvironment through exosome secretion. Either engrafted AML cells or AML-derived exosomes increased mesenchymal stromal progenitors and blocked osteolineage development and bone formation *in vivo*. Pre-conditioning with AML-derived exosomes "primed" the animals for accelerated AML growth. Conversely,disruption of exosome secretion in AML cells through targeting Rab27a, an important regulator involved in exosome release, significantly delayed leukaemia development. In BM stromal cells, AML-derived exosomes induced the expression of DKK1, a suppressor of normal hematopoiesis and osteogenesis, thereby contributing to osteoblast loss. Conversely, treatment with a DKK1 inhibitor delayed AML progression and prolonged survival in AML-engrafted mice. In addition, AML-derived exosomes induced a broad downregulation of hematopoietic stem cell supporting factors (e.g., CXCL12, KITL, and IGF1) in BM stromal cells and reduced their ability to support normal hematopoiesis. Altogether, this study uncovers novel features of AML pathogenesis and unveils how AML cells create a self-strengthening leukaemic niche thatpromotes leukaemic cell proliferation and survival, while suppressing normal hematopoiesis through exosome secretion.

OS27.05

Bio-engineered extracellular microvesicles and cell membrane-derived nanoghosts as multivalent approach for cancer therapy

Vladimir Mulens-Arias[1], Alba Nicolás-Boluda[2], Alice Balfourier[2], Amanda Brun[2], Claire Wilhelm[2], Florent Carn[2] and Florence Gazeau[2]

[1]Laboratoire Matières et Systèmes Complèxes; [2]Laboratoire Matière et Systèmes Complexes, UMR 7075, CNRS and Université Paris Diderot

Introduction: In physiological and altered conditions, cells shed submicronic extracellular vesicles (EVs) containing a variety of soluble and membrane-embedded factors. All of them mediate intercellular communication, thereby allowing cross-regulation. The induction of antitumour immune response is one of the more extended strategies for cancer treatment. In addition, cell membrane-derived vesicles produced by physical extrusion, known as nanoghosts, harbour enormous potential as therapeutic platform. We then intend to use both types of membrane-based vesicles as drug nanocarriers for cancer theranosis.
Methods: We started the characterisation of empty or SPION-loaded EVs derived from different cell types as to their immunogenicity and effects on podosome/invadosome dynamics. We produced cell membrane nanoghosts loaded with 5-fluorouacil and gold nanoparticle and tested their potential as photothermal and photoacoustic agents.
Results: EVs derived from mouse colon carcinoma CT26, mouse endothelial cells SVEC, mouse mesenchymal stem cells, and HUVEC activated macrophages as determined by cytokine secretion and modulated podosome dynamics. Importantly, SPION-loaded EVs appeared to modulate macrophage behaviour in a different manner as compared to empty EVs. We loaded nanoghosts with gold nanoparticles and fluorouracil and we proved their plasmonic behaviour for photothermal therapy and photo-acoustic imaging purposes. This all-in-one biocamouflaged platform combines photothermia, drug-loading and immune-modulation functions to tackle tumour cells by multiple fronts while enabling imaging follow-up.
Conclusions: Altogether, EVs might potentiate natural or induced immune response suggesting their use as nanocarriers in combinatorial therapeutic approaches. Likewise, cell membrane-derived gold nanoparticle-loaded nanoghosts show promising properties for their exploitation in cancer multivalent therapy.

Poster Session S01 – EVs and Stem Cells II
Chairs: TBD and Yaxuan Liang 5:15–6:30 p.m.

PS01.01

PPARα carried by microparticles restores the failed differentiation and functionality of bone marrow-derived cells induced by high-fat diet

Luisa Vergori[1], Emilie Lauret[2], Raffaella Soleti[2], Ramaroson Andriantsitohaina[1] and M. Carmen Martinez[1]

[1]INSERM U1063; [2]INSERM UMR1063 – University of Angers, France

Metabolic pathologies such as diabetes and obesity are associated with decreased level of circulating and bone marrow (BM)-derived endothelial progenitor cells (EPCs). It is known that activation of peroxisome proliferator-activated receptor (PPARα) may stimulate cell differentiation. In addition, microparticles (MPs), small membranes vesicles produced by activated and apoptotic cells, are able to reprogram EPCs. Here, we evaluated the role of PPARα carried by MPs on both phenotype and function of progenitor cells from mice fed with a high-fat diet (HFD). Male (C57BL/6N, 8 weeks-old) mice received either a standard or a high-fat diet (HFD) (42% kcal from fat) for 12 weeks. Bone marrow (BM)-derived cells were obtained from femurs and tibias of mice and cultured in the absence or in the presence of MPs taken either from wild-type (PPARα+/+) or PPARα knock out (PPARα-/-) mice for 7 days. Characterisation of cells was performed by flow cytometry. The effects of MPs *in vivo* neovascularisation were studied by Matrigel plug assay. We observed that HFD induced hyperglycemia and dyslipidemia, and reduced circulating EPCs. After 7 days of culture, BM-derived EPCs and monocytic progenitor cells from HFD-fed mice displayed impaired differentiation. At the same time, we show that MPs bearing PPARα, $MPs^{PPARα+/+}$, increased the differentiation of EPCs and monocytic progenitors from HFD-fed mice, whereas $MPs^{PPARα-/-}$ had not effect on the differentiation of all types of progenitor cells. Furthermore, $MPs^{PPARα+/+}$ increased the ability of progenitor cells to promote *in vivo* angiogenesis in mice fed with HFD. The *in vitro* and *in vivo* effects of $MPs^{PPARα+/+}$ were abolished in presence of PPARα inhibitor, MK886. These data highlight the ability of PPARα carried by MPs to restore the failed differentiation and functionality of BM-derived cells induced by HFD.

PS01.02

Divergence of glioblastoma stem cell phenotypes during *in vivo* development of resistance to temozolomide is reflected by cargo of extracellular vesicles

Delphine Garnier[1], Brian Meehan[2], Laura Montermini[2], Thomas Kislinger[3], Ichiro Nakano[4] and Janusz Rak[3]

[1]UMR Inserm 892/CNRS 629 – CRCNA Nantes; [2]The Research Institute of the McGill University Health Center, Montreal, Canada; [3]Princess Margaret Cancer Center, Toronto, Canada; [4]Department of Neurosurgery, University of Alabama at Birmingham, AL, USA

Introduction: Glioblastoma multiforme (GBM) represents the most frequent and almost uniformly fatal class of grade IV (WHO) primary astrocytic brain tumours, and is associated with the median survival of only 12–15 months post diagnosis. Therapy combines surgical resection, radiation and adjuvant courses of oral temozolomide (TMZ), unfortunately the initial response is followed by acquisition of resistance by GBM stem cells (GSCs). To better detect, understand and prevent the occurrence of resistance to TMZ chemotherapy, we investigated the profile of extracellular vesicles (EVs) secreted by TMZ-sensitive and -resistant GSCs from the Mesenchymal GBM subtype.
Methods: We generated GBM xenografts through orthotopic implantation of human mesenchymal GSCs into NSG mice. While the control group was left untreated, the other mice were treated with several rounds of TMZ, leading initially to tumour response but eventually to the acquisition of resistance by GBM cells, and fatal tumour relapse. EVs were purified from both TMZ-sensitive and -resistant GSC lines, and analysed by nanoparticle tracking analysis (NTA) and mRNA expression profiles.
Results: Individual tumours derived from the same isogenic GSC line expressed divergent profiles of TMZ resistance markers, with a minor representation of the O6-methyl guanine DNA methyltransferase (MGMT). The changes in mRNA profiles, reflective of TMZ resistance and stemness expressed by chemo-resistant GSCs, were recapitulated in the transcriptome of exosome-like EVs released by these cells into the culture medium. Moreover a significant increase in the number of EVs released was observed in 2 over 3 TMZ-resistant variants compared to TMZ-sensitive GSCs.
Conclusion: Thus, GBM tumour initiating cells harbour multiple alternative programmes that translate into chemotherapy resistance *in vivo*, and can be monitored by molecular profiling of stem cell-related EVs.

PS01.03

Promising effects of menstrual blood mesenchymal stromal cell exosomes on inflamation in wound healing process of diabetic mice

Razieh Dalirfardouei, Khadije Jamialhmadi and Elahe Mahdipour

Department of Medical Biotechnology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Introduction: Wound healing is a complicated process that contains some overlapping and consecutive phases including inflammation, proliferation and remodelling. Disruption in each phase can cause chronic non-healing wounds. Most of the chronic wounds do not respond to common therapeutic procedure. Currently, there is a growing interest to use mesenchymal stem cells (MSCs) especially their paracrine factors to improve wound healing process. The focus of the recent researches has been on exosomes as paracrine factors derived from MSCs. These natural nanovehicles contain bioactive macromolecules which affect intracellular signalling pathways similar to MSCs without their detrimental effects. In the current study, we investigated the effects of exosomes released from menstrual blood-derived MSCs on wound healing in diabetic mice.
Methods: MSCs derived from menstrual blood were characterised by flow cytometry and differentiation potential. The exosomes were isolated from conditioned media using ultracentrifugation and were characterised by AFM, TEM and western blotting for CD81 and TSG101. The exosomes were quantified by ELISA. A full thickness excisional wound was created on the dorsal skin of each STZ-induced diabetic C57BL/6 male mice. Eighteen mice were divided into three groups as follows: PBS group, exosomes group (10 μg) and MSC group (1×10^6 cells). The wound tissues were excised on day four to evaluate the inflammation process through iNOS (as M1 marker) and arginase (as M2 marker) activity assay and RelA gene expression.
Results: To evaluate the effects of exosomes on macrophages, iNOS and arginas enzyme activities were measured. In the exosome group, iNOS activity was significantly decreased compared with MSC group and control group. However, we did not observe any significant increase in arginase activity. The expression level of RelA was assessed to evaluate the NFκB as a well-characterised pro-inflammatory signalling pathway. The RelA gene expression was remarkably decreased in exosome group($p < 0.05$)which was assessed with real time PCR.
Conclusion: The results showed that the exosomes preferentially lead to M1/M2 polarisation as well as the decrease in RelA expression comparing to MSCs. Conclusively, exosomes can ameliorate wound healing through inflammation reduction.

PS01.04

Convective exosome-tracing microfluidics for analysis of cell-non-autonomous neurogenesis

Do Won Hwang[1], Hyun Jeong Oh[1], Hyunjong Lee[1], Yoojin Shin[2], Dong Soo Lee[1] and Seok Chung[2]

[1]Department of Nuclear Medicine, Seoul National University, Seoul, Republic of Korea; [2]School of Mechanical Engineering, Korea University, Seoul, Republic of Korea

Introduction: The effective role of exosome delivering neurogenic microRNA (miRNA) enables to induce efficient differentiation process during neurogenesis. The microfluidic system capable of visualising the exosomal behaviour such as secretion, migration, and uptake of individual exosomes can be used as a robust technique to understand the exosome-mediated change of cellular behaviour. Here, we developed the exosome-tracing microfluidic system to visualise exosomal transport carrying the neurogenic miRNA from leading to neighbouring cells, and found a new mode of exosome-mediated cell-non-autonomous neurogenesis.
Methods Exosomes were visualised using GFP-tagged CD63 plasmid vector. Live-cell imaging was performed by confocal microscopy on microfluidic device. NE-4C, neural stem cells and F11, neural progenitor cells were used to monitor exosomal behaviour. To detect miRNA expression, pRV-effLuc/3xPT_miR-193a vector containing the triplicates of miRNA binding site in the 3′ UTR of effLuc was used.
Results The miR-193a facilitated neurogenesis in F11 cells by blocking proliferation-related target genes. In addition to time-lapse live-cell imaging, microfluidics system visualised the convective transport of exosomes from differentiated to undifferentiated cells. Individual exosomes containing miR-193a from differentiated donor cells were taken up by undifferentiated cells to lead them to neurogenesis. Induction of anti-miR-193a was sufficient to block neurogenesis in F11 cells. Inhibition of the exosomal production by manumycin-A and treatment of anti-miR-193a in the differentiated donor cells failed to induce neurogenesis in undifferentiated recipient cells.
Conclusions These findings indicate that neural progenitors and neurogenic miRNA within exosomes propagate cell-non-autonomous differentiation to neighbouring progenitors, and delineate the roles of exosome mediating neurogenesis of population of homologous neural progenitor cells

PS01.05

Mechanisms of exosomal secretion of Wnt proteins

Alena Ivanova[1], Oksana Voloshanenko[1], Jan Winter[1] and Michael Boutros[1,2]

[1]German Cancer Research Centre (DKFZ), Division Signalling and Functional Genomics; [2]Heidelberg University, Department of Cell and Molecular Biology, Faculty of Medicine Mannheim, Heidelberg, Germany

The Wnt signalling pathway plays an important role during development, carcinogenesis and many other diseases. According to the current understanding of Wnt secretion, Wnt proteins are palmitoylated by the membrane-bound O-acyltransferase Porcupine in the endoplasmic reticulum (ER) and then transported into the Golgi by p24-mediated sorting into COPII-vesicles. Subsequently, the cargo receptor Evi/Wls is responsible of the intracellular movement and secretion of Wnt proteins: it binds Wnt proteins in the ER and transports them to the plasma membrane. Previously, we have shown that Wnt proteins can be recycled through the endosomal compartment and secreted on exosomes (1). However, the mechanisms how Wnt proteins are secreted on exosomes as well as general factors required for exosomal release remain largely unknown.
Here, we established genetic tools to identify genes which are involved in the secretory pathway of Wnt proteins. We use CRISPR/Cas9 screening technologies for targeted disruption of genes in combination with Wnt activity assays to identify genes that are required for the secretion of functional Wnt proteins. A panel of 50 candidate secretory factors genes have been we have identified several genes with a potential regulatory role in Wnts secretory pathway. In summary, the established tool will contribute towards the understanding of Wnts trafficking and their secretion routes.

Reference

1. Gross et al., *Nat. Cell Biol.* 2012; 14, 1036–1045.

PS01.06

Extracellular vesicles from adipose-derived mesenchymal stem cells increase the phagocytic activity in peritoneal macrophages

Carmen Carceller[1], Isabel Guillen[1,2], Alba Martinez[3], Maria Luisa Gil[3], Maria Luisa Ferrandiz[1] and Maria Jose Alcaraz[1]

[1]IDM, University of Valencia, Spain; [2]Department of Pharmacy, CEU-Cardenal Herrera, Valencia; [3]Department of Microbiology and ERI BIOTECMED, University of Valencia, Spain

Introduction: The secretome from adipose tissue derived mesenchymal stem cells (ASC) has been shown anti-inflammatory and immunomodulatory activity in different conditions. However, the contribution of extracellular vesicles, microvesicles (Mv) and exosomes (Ex) to the effects of ASC secretome, has not been widely studied.The purpose of this work was to investigate whether Mv and Ex from ASC can regulate the phagocytic activity and the production of inflammatory mediators during the innate immune response in mouse peritoneal macrophages.
Methods: CD1 male mice were used to isolate macrophages from the peritoneal cavity and ASC from perigonadal fat pads. Isolation of Ex and Mv from ASC secretome was performed by differential (ultra) centrifugation combined with size filtration. Tunable resistive pulse sensing was used to evaluate the concentration and size of Ex and Mv. After characterisation of macrophages by flow cytometry, they were seeded and stimulated with lipopolysaccharide (LPS, 1 μg/ml), and treated with 2×10^7 Ex/ml or 9×10^4 Mv/ml for 20 h. Phagocytosis assay was performed by flow cytometry and confocal microscopy, IL-1β, TNFα and KC production was measured by ELISA, PGE_2 by RIA and nitrite levels by fluorometry. The data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.
Results: The secretion of inflammatory mediators and the phagocytic activity of macrophages were significantly increased after LPS stimulation compared with cells in basal conditions. Ex significantly reduced the levels of TNFα, PGE_2 and NO with respect to the LPS control whereas Mv only diminished TNFα. Regarding the phagocytic activity, both Ex and Mv raised it significantly. Our data suggest that extracellular vesicles can regulate macrophage activity in the innate immune response and contribute to the anti-inflammatory effects of ASC. These findings support the interest of Ex for the development of potential new approaches to the treatment of inflammatory diseases.

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PS01.07

Role the central carbon metabolism pathway in tumour stromal support – a study using extracellular vesicles of mesenchymal stem cells from normal and osteosarcoma participants

Patrice Penfornis[1], K. Sreekumaran Nair[2] and Radhika Pochampally[1]

[1]University of Mississippi Medical Center, MI, USA; [2]Mayo Clinic, Rochester, MN, USA

Introduction: Previous studies have shown the role of mesenchymal stem cells (MSCs) from bone marrow in the growth and metastasis of solid tumours but mechanisms remains unclear in osteosarcoma (OS). Our recent study have shown the role of MSCs extracellular vesicles (EVs) in the proliferation and migration of osteosarcoma cells (PMID27812189). We have previously characterised MSCs-EVs using genomics, lipidomics and proteomics (PMID25669974). In this study we focused on difference in the metabolome of EVs from MSCs from healthy and diagnosed OS participants.
Methods: Biopsies from cancer participants were collected and osteosarcoma along with bone marrow mesenchymal stem cells were derived in cell culture. Cell phenotypes were confirmed using specific markers. Normal MSCs from healthy patient has been used as reference. All cells were cultured in factories (>10^8 cells) and serum-free cell supernatant were collected, concentrated and extracellular vesicles were isolated using serial ultracentrifugation. Purified EVs were analysed at the

Mayo Clinic Metabolomics Core. In parallel, cells mRNA levels were analysed by microarray at the UMMC Genomics Core. Data was normalised and analysed by one-way ANOVA and integrated molecular pathway level analysis.
Results: Preliminary data showed that EVs studied contains at least 250 metabolites with a KEGG ID. Ontology revealed an enrichment of metabolites involved in arginine and proline degradation pathways. Compared to their OS-EVs, cancer patient MSCs-EVs are, for example, enriched in adenine and hexanoylglycine (20–40 fold). Interestingly, cancer patient MSCs-EVs are notably enriched of succinic acid, lactic acid, proline, phosphoenol pyruvate, fumaric acid (3–10 fold range) compared to the healthy patient-derived MSCs-EVs. Correlation between metabolites and gene expression up-regulation revealed the involvement of the central carbon metabolism in cancer pathway (KEGG hsa05230). Moreover, proteomics data showed that 9 glycolysis pathway enzymes are detected in MSCs-EVs.
Conclusion: This preliminary study reveals that cancer patient MSCs secrete EVs which are enriched with metabolites that are in demand by OS cancer cells metabolism, thus promoting tumour growth. These data confirm multiple supportive roles of stromal cells EVs in cancer progression.

PS01.08

Cardiosphere-derived cell and mesenchymal stem cell extracellular vesicles contain distinct RNA cargo

Ann-Sophie Walravens, Kiel Peck, Linda Marban and Geoffrey de Couto and Luis Rodriguez-Borlado

Capricor Therapeutics

Introduction: Cardiosphere-derived cells (CDCs) possess regenerative, immunomodulatory and cardioprotective characteristics when delivered to the heart post-myocardial infarction (MI). In contrast to other cell types, this vast array of therapeutic benefits appears to be a unique trait to CDCs. We've previously demonstrated that human CDC-derived extracellular vesicles (CDC-EVs) recapitulate the effects of CDCs in acute and chronic *in vivo* models of MI. Thus, we believe that the efficacy of CDC therapy is mediated by CDC-EVs. Here we are testing the hypothesis that a distinct cargo profile will define the functional efficacy between CDC-EVs and mesenchymal stem cell-derived EVs (MSC-EVs).
Methods: CDCs or MSCs were cultured in serum-free medium for 15 days and then the conditioned media was concentrated by ultrafiltration (MWCO 10 kDa) to isolate EVs. The miRNeasy Serum/Plasma kit (QIAGEN) was used to extract total RNA from EVs followed by small RNA sequencing (NextSeq 500, Illumina) (CDC-EVs, $n = 5$, MSC-EVs, $n = 2$) or nCounter miRNA expression analysis (Nanostring technologies).
Results: RNA sequencing analysis of CDC-EVs and MSC-EVs revealed a greater overall abundance of Y-RNA fragments in CDC-EVs. When we examined the specific Y-RNA classes, we found that MSC-EVs contained an expression profile with lower Y4 ($p < 0.05$), but higher Y5 ($p < 0.05$), Y-RNA fragments. Interestingly, miR-22 was highly expressed in both CDC-EVs and MSC-EVs, which suggests that it may serve as a housekeeping miRNA for EVs derived from different cell sources. Four miRNAs (miR-146a, miR-151, miR-409, miR-423) were highly enriched in CDC-EVs, while miR-10a and miR-4792 were more abundant in MSC-EVs. These miRNA results are being validated by nCounter miRNA expression analysis.
Conclusion: Here, we've demonstrated that CDC-EVs contain a unique RNA cargo set that can differentiate CDC-EVs from MSC-EVs. The higher presence of Y RNA in CDC-EVs as compared to MSC-EV could be responsible for the ability of CDCs to regulate stem cell activation and tissue regeneration. The highly enriched panel of miRNAs observed in CDC-EVs, in contrast to MSC-EVs, suggest that they may support some of the functional benefits observed post-MI.

PS01.09

Enhanced cardiomyogenic and angiogenic potential of extracellular vesicles derived from genetically modified stem cells expressing selected micro RNAs

Sylwia Bobis-Wozowicz[1], Katarzyna Kmiotek[1], Malgorzata Sekula[2], Dariusz Boruczkowski[3], Jacek Kolcz[4], Zbigniew Madeja[1] and Ewa K. Zuba-Surma[5]

[1]Jagiellonian University, Krakow, Poland; [2]Malopolska Centre of Biotechnology; [3]Polish Stem Cell Bank, Poland; [4]Polish-American Children's Hospital, Poland; [5]Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

Introduction: Extracellular vesicles (EVs) are small circular structures composed of a cellular membrane and cytosolic cargo, mainly small RNAs, proteins and lipids. By transferring their bioactive components to other cells, EVs may influence cell fate and behaviour. This study investigated efficacy of EVs isolated from human umbilical cord-derived mesenchymal stem cells (hUC-MSC) and human induced pluripotent stem cells (hiPSC) in transferring selected proangiogenic and cardiomyogenic miRNAs (miR-1, miR-199a and miR-126) to cardiac mesenchymal stromal cells (cMSC).
Methods: hUC-MSCs and hiPSCs were genetically engineered to express selected miRNAs and green fluorescent protein (copGFP) by lentiviral transduction. EVs were isolated by sequential ultracentrifugation (2000*g*, 100,000*g* ×2) from cell conditioned media. Expression of the transgenes were analysed in EVs, their parental cells and cMSCs by real time qPCR. cMSC upon EVs transfer were subjected to cardiomyogenic and angiogenic differentiation.
Results: Genetic modification of hUC-MSC and hiPSC resulted in constitutive and stable expression of selected miRNAs in these cells, which was confirmed by molecular analyses. EVs isolated from these cells contained elevated levels of the introduced miRNAs. EVs cargo was efficiently transferred to the acceptor cells – cMSCs – and enhanced their differentiation towards cardiac and endothelial lineages.
Conclusion: In this study we have shown that EVs isolated from genetically modified hiPS and hUC-MSC were enriched in specific miRNAs, which modulated ability of target cells to differentiate into cardiac and endothelial lineages. Obtained results indicate usefulness of stem cells-derived EVs as potential tools in miRNAs transfer, which can be further exploited in regenerative medicine.

PS01.10

Cell-engineered nanovesicle as a surrogate inducer for contact-dependent stimuli

Chungmin Han, Junho Kim, Yong Song Gho and Jaesung Park

Pohang University of Science and Technology, Pohang, Republic of Korea

Heterotypic interactions between cells are crucial in various biological phenomena. Particularly, stimuli that regulate embryonic stem cell (ESC) fate are often provided from neighbouring cells. However, except feeder cultures, there have been no practical methods that can provide ESCs with contact-dependent cell stimuli. To induce contact-dependent cell stimuli in the absence of living cells, we describe a new method that uses cell-engineered nanovesicles (CNV) that are made by extruding living cells through micro-porous membrane. Protein compositions of CNVs are similar to their originating cells, and freely diffusible and precisely scalable. Treatment of CNVs produced from three different stromal cells successfully induced the same effect as feeder cultures. Our results suggest that the effect of CNVs are mainly mediated by membrane-associated components. The use of CNVs might provide us with a new and efficient tool for ESC research.

PS01.11

Economics and quality attributes of hMSC production in xeno-free bioprocessing media

Lye Theng Lock, Timothy Olsen, Keith Dailey and Jon Rowley

RoosterBio

Human mesenchymal stem cells (hMSCs) are key raw materials in Regenerative Medicine and are widely used for cell or secretome-based therapeutics, engineered tissues, or medical devices. Yet, achieving an economical bioprocess for hMSC production remains a significant challenge in the regenerative medicine industry. Modelling of hMSC manufacturing bioprocess economics identifies culture media as a major cost

driver in a cell manufacturing process. The availability of efficient and robust xeno-free bioprocessing media will not only reduce manufacturing cost, but also decrease regulatory burden associated with bovine serum components found in traditional culture media. Here, we evaluated and compared hMSC quality parameters in bovine serum-containing and xeno-free bioprocess media formulations and assessed quality parameters such as cell identity and potency. The hMSCs cultured in xeno-free media expanded rapidly and achieved confluency within 4–5 days without media exchange in 2D culture. Furthermore, cells in xeno-free media maintained critical hMSC functional properties including angiogenic cytokine (FGF, HGF, IL8, TIMP1, TIMP2 and VEGF) secretion, trilineage differentiation, and immunomodulatory potential. A scale-up study using a 3D bioreactor platform with a xeno-free bioreactor feed was conducted and was determined to be a robust scalable production format for hMSCs. The economics of hMSC expansion in this xeno-free media system were modelled for both 2D and 3D bioreactor culture, and the critical productivity metric of million cells generated per litre of media demonstrated that the xeno-free media consistently outperformed traditional hMSC media systems (by yielding more than 8-fold higher cells/L media used), thus making it the robust and economic choice for industrial-scale manufacturing of hMSCs for its secretome or cellular material.

LBP.43

A comparison of exosome isolation methods from conditioned media of amniotic fluid stem cells

Lina Antounians, Vincenzo Catania, Adrienne Sulistyo, Alison Hock, Bo Li and Augusto Zani

The Hospital for Sick Children

Introduction: Amniotic fluid stem cells (AFSC) are CD117+ cells that express markers of pluripotency, and can be directed into all three primary embryonic cell lineages. AFSC have proved to be effective in tissue regeneration in various disease models, mainly acting through a paracrine mechanism. There has been an increasing interest in determining the potential of AFSC-derived exosomes as an alternative treatment option to cell-based therapy. The aim of our study was to compare isolation methods of exosome derived from AFSC conditioned media, that to the best of our knowledge has not yet been established.

Methods: Conditioned media was collected from AFSC grown overnight in exosome-depleted media. 2mL was used to compare exosome isolation methods via ultracentrifugation, Exo-Prep, Exo-Quick, and Total Exosome Isolation reagents, following supplier recommended protocols (at least three replicates per method). Exosome protein was quantified using the Pierce Bradford assay. Exosomes were visualized under transmission electron microscopy and assessed for vesicle size via NanoSight. Exosomes were tested for protein expression via Western blot using markers CD9, CD63, CD81, Hsp70, and negative markers for Histone H3.

Results: Exosomes isolated from all methods were positive for CD63 and Hsp70 markers, but did not show detectable levels of CD9, CD81 via Western blot. Exo-Prep reagent precipitated the highest protein concentration compared to ultracentrifugation and other commercially available kits. Ultracentrifugation yielded the highest concentration of CD63 and Hsp70 protein. Nanoparticle tracking revealed that Total Exosome Isolation reagent had the highest yield of nanoparticles within 30-120nm range.

Summary/Conclusion: Given the low cost and high purity obtained using ultracentrifugation, this is our preferred method of exosome isolation from AFSC. Further studies are needed to assess the reparative effects of AFSC derived exosomes isolated from these different methods.

Funding: SickKids Start-Up Fund.

Poster Session S02 – EVs for Therapeutic Applications
Chairs: Mario Gimona and Andre Gorgens 5:15–6:30 p.m.

PS02.01

Evaluation of cellular uptake of exosomes during cancer treatment with gefitinib

Tomoya Takenaka[1], Miku Katayama[1], Ikuo Fujii[1], Susumu Kobayashi[2] and Ikuhiko Nakase[1]

[1]Osaka Prefecture University, Osaka, Japan; [2]Beth Israel Deaconess Medical Center/Harvard Medical School, MA, USA

Introduction: During cell-to-cell communication, extracellular vesicles (EVs) such as exosomes play crucial roles because they deliver biofunctional molecules (e.g. microRNAs and enzymes) into cells that control cellular functions. In cancer progression, exosomal communications have been shown to participate significantly. Therefore, it is important to understand the effects of cancer treatment on exosomal communications. Gefitinib (commercial name: Iressa) is a tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR) and is approved for the therapeutic treatment of non-small cell lung cancers (NSCLCs) with EGFR mutations. In this study, we demonstrated the influence of gefitinib on cellular exosome uptake and cancer treatment.
Methods: HCC827 (mutant EGFR) and A549 (wild-type EGFR) cells, which are gefitinib-sensitive and low-sensitive NSCLCs, respectively, were treated with FITC-dextran-loaded exosomes (derived from HeLa cells) or fluorescein-labelled liposomes in the presence or absence of gefitinib (10 nM), the concentration of which did not affect cell growth under this experimental condition. After 24 h incubation, each cellular-uptake efficacy was assessed using flow cytometry and confocal microscopy. Moreover, the cytotoxicity of doxorubicin (DOX)-loaded exosomes or liposomes on the cells in the presence or absence of gefitinib was also assessed using OneCell counter.
Results and Conclusion: In HCC827 cells, the cellular uptake of exosomes was enhanced, while that of liposomes was suppressed by gefitinib treatment, suggesting that the cellular uptake pathways for exosomes and liposomes are different in gefitinib-sensitive HCC827 cells. On the contrary, no change was observed in gefitinib-low-sensitive A549 cells. The same trend was observed in the cytotoxicity study. The gefitinib treatment enhanced the cytotoxicity of DOX-loaded exosomes, but inhibited the cytotoxicity of DOX-loaded liposomes. Moreover, DOX-loaded exosomes showed superior anti-cancer efficacy compared to DOX-loaded liposomes based on IC_{50}. These findings indicate that exosomal cell-to-cell communication is possibly affected by cancer treatment with gefitinib, and exosome-based intracellular delivery is considered to have pharmaceutical advantages.

PS02.02

Enzymatic exosomes with GPI-anchored hyaluronidase for enhanced tumour penetration and anti-tumour efficacy

Yeon-Sun Hong[1], Yoosoo Yang[2] and In-San Kim[2]

[1]KU-KIST Graduate School of Converging Science and Technology, Korea University; [2]Korea Institute of Science and Technology

Please see OPT01.05

PS02.03

Efficient delivery of glucocerebrosidase lysosomal enzyme via EXPLOR technology for treatment of Gaucher disease

Hojun Choi[1], Kyungsun Choi[1], Nambin Choi[1], Seung Wook Choi[2] and Chulhee Choi[1]

[1]KAIST, Seoul, Republic of Korea; [2]Cellex Life Sciences, Inc

Introduction: We have previously developed an opto-genetically engineered exosome system, named "exosomes for protein loading via optically reversible protein–protein interaction" (EXPLOR) that can deliver soluble proteins into the cytosol via controlled, reversible protein–protein interactions (PPI). Treatment with protein-loaded EXPLORs was shown to significantly increase intracellular levels of cargo proteins and their function in recipient cells in both a time- and dose-dependent manner. In the present study, we tested the feasibility of EXPLOR technology for delivery of beta-glucocerebrosidase (GBA) as a potential treatment for Gaucher disease.
Methods: In the present study, we have incorporated GBA enzyme into the engineered exosomes by fusion with optically controlled PPI module. GBA-loaded exosomes were then tested for protein loading efficiency and *in vitro* enzymatic activity. Patient-derived fibroblasts were tested for delivery of GBA by GBA-loaded exosomes.
Results: We were able to load GBA into engineered exosomes by transiently or stably expressing fusion proteins in exosome producing cells. We further demonstrated the intracellular delivery of GBA as functional proteins in the target cells *in vitro* and target organs *in vivo*.
Conclusion: These results clearly indicate the potential of EXPLORs for treatment of Gaucher disease.

PS02.05

Intein mediated enrichment of soluble proteins into exosomes

Justin Hean[1], Imre Mäger[2], Inna Uliyakina[1], Joel Z. Nordin[3], Samir EL-Andaloussi[3,2] and Matthew J. Wood[2]

[1]University of Oxford, Oxford, UK; [2]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK; [3]Department of Laboratory Medicine, Karolinska Instiutet, Stockholm, Sweden

Introduction: Exosomes are readily taken up by many cell types, in what appears to be an energy-dependent, directed process. Furthermore exosomes have been described to transport a variety of bioactive molecules such as proteins, lipids and nucleic acids. Together with their possible non-immunogenic properties, exosomes facilitate a new paradigm in the delivery of therapeutic agents. However, owing to their biogenesis mechanisms, exosomes are not readily enriched with targeted soluble proteins without anchoring to the exosomal membrane or incurring bulky fusion modifications. Here we demonstrate that utilising an exosomal co-localisation signal, and a self-cleaving protein we are able to enrich for specific soluble proteins within the exosomal lumen.
Methods: DNA constructs were generated by introducing the self-cleaving intein, ΔIC-TM, downstream of the CD63 ORF. Following the intein region a reporter ORF of interest was inserted. Exosomes containing the self-cleaving constructs were generated in HEK293t cells and characterised by NTA and western blotting. Finally the reporter enriched exosomes were co-incubated with recipient cell lines and analysed by confocal microscopy or appropriate readout assay.
Results: Here we show that exosomes are successfully enriched with the reporter protein, independent of the co-localisation signal-intein fusion. NTA and western blot analysis of the vesicles suggests little to no variation from their wild type counterparts. Finally, exosomes enriched with the reporter proteins are readily taken up by recipient cells, and display evidence of cargo protein assimilation
Conclusion: Here we describe a novel method of enriching exosomes with a soluble protein independent of remnant co-localisation fusions. These enriched exosome were demonstrated to deliver their cargo to recipient cells. We envisage this strategy applicable to both basic and therapeutic biology alike.

PS02.06

Delivery of membrane-bound CD39/CD73 by extracellular vesicles (EVs) for treatment of inflammatory disease

Susanne A. Snoek[1], Niels Broekstra[1], Jan van Ittersum[1], Jeroen de Vrij[2], Edwin van der Pol[3], Rienk Nieuwland[4], Lisa G.M. van Baarsen[5], Paul P. Tak[1], Margriet Vervoordeldonk[1] and Jonathan Finn[1]

[1]Arthrogen BV; [2]Department of Neurology, Erasmus Medical Center; [3]Biomedical Engineering & Physics and Vesicles Observation Center, Academic Medical Center; [4]Clinical Chemistry department, Academisch Medisch Centrum; [5]Clinical Immunology and Rheumatology, Academic Medical Center

Introduction: Our recent data demonstrated that the balance between pro-inflammatory extracellular ATP and anti-inflammatory adenosine is skewed in the synovial compartment of rheumatoid arthritis patients, likely contributing to ongoing inflammation. CD39 is an ATPase that converts ATP and ADP into AMP, while CD73 converts AMP into adenosine. CD39 and CD73 are membrane bound enzymes and previous studies have shown that removing the transmembrane domain of CD39 reduces its activity by >90%. Thus we assessed the potential of extracellular vesicle (EV)-mediated delivery of these membrane-bound enzymes as a novel treatment for inflammatory disease.
Methods: We performed a large scale purification (~50 L) of CD39/CD73-EVs from the supernatant of a stably transfected HEK293 cell line overexpressing both CD39 and CD73. EVs were concentrated by tangential flow filtration, then precipitated using total exosome isolation buffer, and subsequently purified by size exclusion chromatography. Particle concentration was determined using nanoparticle tracking analysis and total protein levels where measured with a micro BCA protein kit. CD39 and CD73 activity of EVs was measured using the malachite green phosphate detection kit, and CD39 or CD73 protein levels were assessed by Western blot.
Results: Purified EVs were very pure as indicated by a high particle/total protein (μg) ratio (5.76E10). Specific enzymatic activity (released phosphate/min/μg protein) of CD39/CD73-EVs was 20.5-fold (CD39) and 4.5-fold (CD73) higher when compared with their soluble counterparts, likely due to maintaining the native structure of the enzymes. CD39/CD73-EVs were ~10-fold more potent in reducing pro-inflammatory cytokine production in *in vitro* human cell-based inflammation assays.
Conclusion: Engineered EVs are a promising tool to deliver membrane-bound, biologically active therapeutic enzymes and may have great potential for the treatment of inflammatory disease, including rheumatoid arthritis.

PS02.07

Synthetic lipid nanoparticles for combination treatment of prostate cancer

Roy van der Meel[1,2], Sam Chen[1,3], Josh Zaifman[1,4], Joslyn Quick[1], Raymond M. Schiffelers[2], Marco A. Ciufolini[1], Yuen Yi C. Tam[1,3] and Pieter R. Cullis[1]

[1]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada; [2]Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, Utrecht, The Netherlands; [3]Integrated Nanotherapeutics, Vancouver, British Columbia, Canada; [4]Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada

Introduction: Current treatment strategies for advanced prostate cancer include androgen receptor (AR) pathway inhibition and taxane-based chemotherapy. However, the effectiveness of chemotherapy is hampered by dose-limiting adverse effects and the vast majority of tumours develop resistance mechanisms against AR inhibitors and taxane drugs. Lipid nanoparticles (LNPs) are the most clinically advanced delivery systems for chemotherapeutics and genetic drugs such as siRNA. Long-circulating LNPs accumulate in tumours to a higher extent compared to free drugs, resulting in increased therapeutic efficacy and reduced adverse effects. We have recently developed new technology that allows the incorporation of virtually any small molecule in LNPs, raising opportunities to combine chemotherapy and gene silencing.
Methods: LNPs containing both taxane chemotherapeutics and siRNA against constitutively active AR variants (AR-V) were formulated by rapid mixing methods. LNPs were characterised by physicochemical analysis including size and drug content. LNP stability in serum was determined by UPLC. Gene silencing efficiency of LNPs was determined by siRNA target knockdown using qPCR. Therapeutic efficacy of LNPs was determined by cell viability assays in PC cell lines expressing AR (variants) including 22Rv1, LNCaP and VCaP while the AR-negative cell line PC3 was used as control.
Results: Physicochemical analysis indicated LNP size of 60 nm and >90% siRNA encapsulation efficiency. The incorporation of taxane chemotherapeutics was varied from 1–10 mol% without effecting the stability of the formulation in serum. LNPs containing siRNA with and without taxane drug induced >80% knockdown of AR-V in 22Rv1 cells. LNPs containing both AR-V siRNA and taxane chemotherapeutics induced greater inhibition of cell viability when compared to control formulations in 22Rv1, LNCaP and VCaP cells while no difference was observed in AR-negative PC3 cells.
Conclusion: LNPs containing both siRNA and chemotherapeutics for are an attractive strategy for the development of effective combination treatments for advanced prostate cancer.

PS02.08

Efficient delivery of super repressor IκB via EXPLOR technology for treatment of chronic inflammatory diseases

Kyungsun Choi[1], Nambin Choi[1], Seung Wook Choi[2], Amin Choi[1], Hojun Choi[1] and Chulhee Choi[1]

[1]KAIST, Seoul, Republic of Korea; [2]Cellex Life Sciences, Inc

Introduction: We have previously developed an opto-genetically engineered exosome system, named "exosomes for protein loading via optically reversible protein–protein interaction" (EXPLOR) that can deliver soluble proteins into the cytosol via controlled, reversible protein–protein interactions (PPI). Treatment with protein-loaded EXPLORs was shown to significantly increase intracellular levels of cargo proteins and their function in recipient cells in both a time- and dose-dependent manner. In the present study, we tested the feasibility of EXPLOR technology for delivery of super repressor IκB (SRI), a potent inhibitor of NF-κB pathway, as a potential treatment for chronic inflammatory diseases such as rheumatoid arthritis.
Methods: In the present study, we have incorporated SRI into the engineered exosomes by fusion with optically controlled PPI module. SRI-loaded exosomes were then tested for protein loading efficiency and *in vitro* inhibitory activity on TNF-α-induced NK-κB activation. Anti-inflammatory effect of SRI-loaded exosomes was tested by systemic administration in a collagen-induced arthritis model.
Results: We were able to load SRI into engineered exosomes by transiently or stably expressing fusion proteins in exosome producing cells. We further demonstrated the intracellular delivery of SRI as functional proteins in the target cells *in vitro* and target organs *in vivo*. Finally, we have observed a beneficial effect of SRI-loaded exosomes in collagen-induced arthritis model compared to naïve exosomes.
Conclusion: These results clearly indicate the potential of EXPLORs for treatment of chronic inflammatory diseases.

PS02.09

Withdrawn at author's request.

PS02.10

Improving extracellular vesicles-mediated mRNA delivery specifically to HER2+ve cancer for effective CNOB/hChrR6 gene-delivered (GDEPT) therapy

Alexis V. Forterre[1], Jing-Hung Wang[1], Alain Delcayre[2], Travis Antes[3], Neil Aronin[4], Anastasia Khvorova[4], Stefanie Jeffrey[1] and A.C. Matin[1]

[1]Stanford University School of Medicine, CA, USA; [2]ExoThera LLC; [3]Cedars-Sinai Medical Centre, Heart Institute, CA, USA; [4]University of Massachusetts Medical School, MA, USA

Introduction: We have been using directed c1c2-anti-HER2 scFv antibody tacked extracellular vesicles (dirEVs) for the delivery of mRNA, encoding the enzyme hChrR6 specifically to HER2+ve cells to confer on them the capacity to activate the prodrug CNOB to generate the cytotoxic agent, MCHB. The latter can be quantified by its fluorescence intensity. Inadequate DNA-mediated gene delivery efficiency and expression compromise the efficacy of GDEPT. We have therefore used mRNA for gene delivery and present here measures for its improved expression.
Methods: We modified the plasmid Xport-MSCV-hChrR6 used previously, by replacing its MSCV promoter by the CMV promoter. The hChrR6 mRNA in EVs and in BT474 recipient cells was quantified by qRT-PCR. EVs functionality was assessed by MCHB fluorescence quantification. Cell viability was assessed by MTT assay.
Results: The use of Xport-MSCV-hChrR6 and CMV-hChrR6 plasmids resulted in the introduction of 0.001 and 0.005 copy of the mRNA per EV, respectively, thus, the latter method decreased the number of EVs required to deliver one copy of the mRNA to the cells from 747 to 196 [73% improvement (p = 0.05)]. MCHB fluorescence in BT474 CNOB-treated cells receiving the mRNA from dirEVs was visible at 24 h. At 30–72 h incubation, BT474 cells receiving mRNA from CMV-hChrR6 dirEVs were 14% more active in generating MCHB than when MSCV-hChrR6 dirEVs was used for mRNA delivery. Cell-free medium of CNOB-treated BT474 cells receiving the mRNA from CMV-hChrR6 dirEVs showed 10 to 20% greater MCHB than cells getting it from MSCV-hChrR6 dirEVs, thus, the use of the former plasmid also generated a stronger bystander effect.
Conclusion: We have improved the engineering of dirEVs containing mRNAs leading to better capacity to activate the prodrug CNOB. Work is in progress to determine the effect at stabilisation of mRNA expression by incorporation of the 3′-UTR of the Beta-globin gene in the upstream region of our mRNA poly (A) tail. Also, we have constructed self-delivery oligos for incorporation into the hChrR6 mRNA to facilitate its passage into the cells and directly into the EVs.

PS02.11

MHC mismatch in exosomal cancer immunotherapy – paving the way for allogeneic exosome treatment?

Pia Larssen[1], Rosanne Veerman[2], Stefanie Hiltbrunner[2], Mikael Karlsson[3] and Susanne Gabrielsson[2]

[1]Karolinska Institutet; [2]Immunology and Allergy Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden; [3]Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, Sweden

Exosomes are interesting as potential cancer immunotherapy vehicles due to their capacity to potentiate immune responses and stimulate tumour-specific immune activation in mice. However, previous clinical trials with peptide-loaded autologous exosomes only showed moderate T cell responses in humans, suggesting that exosome-induced immunity is still not fully understood. We recently demonstrated that antigen-specific $CD8^+$ T cell responses are independent of major histocompatibility complex (MHC) class I presence on exosomes. Furthermore, exosomes lacking MHC class I, as well as exosomes with both MHC class I and II mismatch, are equally efficient in inducing antigen-specific tumour-infiltrating T cells in a B16 melanoma model as autologous exosomes. Still, the effect of multiple injections of allogeneic exosomes has not yet been investigated. We here show that repeated injections of OVA loaded exosomes induce more germinal centre B cells and boost antigen-specific antibody production, thus providing an adjuvant effect *in vivo*. In addition, the effect of repeated injections on tumour clearance in the B16-OVA melanoma model is currently under investigation. In conclusion, our data show that booster injections of allogeneic exosomes result in enhanced antigen-specific $CD8^+$ T cell, germinal centre B cell, and follicular T helper cell responses, as well as increased antigen-specific antibodies. Importantly, our findings support the application of allogeneic exosomes for therapeutic use in humans.

PS02.12

Virus-mimetic fusogenic exosomes for direct delivery of integral membrane proteins to target cell membranes

Yoosoo Yang and In-San Kim

Korea Institute of Science and Technology, Republic of Korea

A number of major human diseases are related to defects in membrane proteins. Despite several efforts aimed at delivering membrane proteins to the defective cell membranes, currently workable approaches to treat such membrane defects have been elusive. Here, we investigated an unprecedented exosome-based nano-platform for delivering membrane proteins directly into recipient cell membranes. Based on the features of exosomes that could be engineered by nature, we developed a fusogenic exosome platform in which expression of the viral fusogen, vascular stomatitis virus (VSV)-G protein, induces the exosomal membrane to fuse with recipient cell membranes at acidic pH. Our results revealed that the fusogenic exosomes could efficiently deliver GFP fused CD63 (CD63-GFP) or glucose transporter-4 (GLUT4-GFP) to recipient cell membrane. Fusogenic exosomes mediated transfer of biologically active GLUT4 to mouse muscle membranes both *in vitro* and *in vivo*, allowing the increased glucose uptake of recipient cells. This highlights the potential of our fusogenic exosome platform for delivering membrane proteins.
(Advanced Materials, Accepted)

PS02.13

Extracellular vesicle-encapsulated oncolytic adenoviruses for enhanced therapeutic effect

Heikki Saari[1], Mariangela Garofalo[1], Petter Somersalo[1], Laura Aksela[2], Elisa Lázaro-Ibáñez[1], Matti Jalasvuori[3], Tatu Rojalin[4], Vincenzo Cerullo[5], Lukasz Kuryk[6] and Marjo Yliperttula[1]

[1]Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland; [2]Orion Corporation; [3]Biological and Enviromental Science, University of Jyväskylä, Finland; [4]University of Helsinki, Finland; [5]Laboratory of ImmunoVirotherapy, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland; [6]Laboratory of ImmunoVirotherapy, Centre for Drug Reserach, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Finland

Introduction: Oncolytic viruses are a promising future treatment option for cancer, however, their use in therapy is limited due to their immune reactivity and requirement towards specific receptors on the surface of the cells to be infected. Here we have studied the possibility of encasing the virus inside extracellular vesicles (EVs) in order to circumvent these limitations by both shielding them from any interactions with immune cells and providing alternative mechanisms for cellular uptake.
Methods: EV-encapsulated oncolytic adenoviruses were prepared by infecting cancer cells with the virus. Once the cells were observed to be dead EVs were isolated from the cell culture medium by ultracentrifugation followed by overnight gradient centrifugation in a linear sucrose gradient. 1 mL fractions were collected from the gradient and analysed by electron microscopy, nanoparticle tracking analysis, blotting for CD9 and adenoviral hexon protein and for their infectivity towards cancer cells.
Results: The EV-encased viruses (V-EVs) were found to migrate to a density characteristic to EVs in the gradient, lower than free viruses. The V-EV fractions were found to be even more infective than the free viruses, acting faster to infect significantly more cells. These fractions were also positive for the viral coat protein but not enriched with CD9, suggesting an apoptotic origin. Unlike free viruses, the V-EVs were also found to retain their infectivity even after treatment with NaOH, suggesting that the EV membrane shields the virus from the treatment.
Conclusion: Infection of cancer cells with oncolytic adenoviruses naturally results in membrane-encased particles containing fragments of the virus. These particles are also more infective and might be effective in the treatment of cancer due to their protective membrane structure. However, an alternative strategy for loading of the viruses inside EVs would be beneficial, since the presented approach applies only to EVs of cancer cells.

PS02.14

Extracellular vesicle-mediated delivery of synthetic receptors for enhanced tumour penetration of targeted agents

Heegon Kim, Chanhee Oh and Ji Ho Park

KAIST, Seoul, Republic of Korea

Introduction: Extracellular vesicles (EV) secreted by cells into extracellular environment mediate intercellular communication by delivering biological materials to neighbouring cells. Since EVs originate from plasma membrane of cells, they also have lipid bilayer structure. In this study, we seek to employ EVs as carriers of synthetic receptors to achieve deep penetration into tumour tissue. Firstly, membrane fusogenic liposomes (MFL) deliver synthetic receptor-lipids (SR-lipid) to tumour cell membrane via membrane fusion. Becoming building blocks of plasma membrane, the synthetic receptor-lipids are incorporated into EVs released by the tumour cells. Hitchhiking the EVs, the synthetic receptors can be further delivered to other cells nearby, spreading throughout tumour regions where conventional liposomes have limited access. Subsequent administration of targeted agents showed tumour-specifically enhanced accumulation and penetration.
Methods: *In vitro*, we examined whether SR-lipids could be localised more selectively onto the plasma membrane of tumour cells than other cells including macrophages, endothelial cells and fibroblasts. In addition, EV-mediated transfer of SR-lipids was inviestigated by isolating EVs from MFL-treated cells and treateing the EVs to other cells. *In vivo*, we intravenously injected MFLs carrying SR-lipids mice bearing 4T1 tumours 1 day prior to intravenous administration of targeted agents. At 2 days, the distribution of targeted agents in tumour sections was studied using a confocal microscope and NIS-elements BR software.
Results: *In vitro*, we observed that SR-lipids were delivered to tumour cell membrane by MFLs. We also observed that the SR-lipids on the cell membrane were further transferred to neighbouring cells by EVs. The SR-lipids provided binding sites on the cells for targeted agents. *In vivo*, we found out that the selective delivery of SR-lipids to tumour cell membranes using MFLs enhanced accumulation and penetration of subsequent targeted agents.
Conclusion: By using liposomes to modify tumour cell membrane and hitchhiking EVs, we successfully delivered synthetic receptors throughout a tumour and achieved enhanced accumulation and penetration of targeted agents.

PS02.15

In vitro insertion of ligands to extracellular vesicles for efficient *in vivo* cancer targeting and regression with little liver accumulation

Fengmei Pi[1], Zhefeng Li[1], Daniel Binzel[1], Bin Guo[2] and Peixuan Guo[1,3]

[1]Division of Pharmaceutics and Pharmaceutical Chemistry, College of Pharmacy; [2]Department of Pharmacological and Pharmaceutical Sciences, School of Pharmacy, Houston, TX, USA; [3]Department of Physiology & Cell Biology, College of Medicine, Dorothy M Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH, USA

Utilisation of extracellular vesicles (EVs) for non-viral gene delivery is an emerging platform in nanotechnology and nanomedicine. Here we report the combination of the advantages of both EV for membrane fusion and nanotechnology for ligand displaying, resulting in specific cancer targeting and cytosol delivery. Using special sedimentation separation technology, our small exosome showed reduce accumulation in liver and other normal organs. Delivery of therapeutic RNA molecules with this new strategy shows significant gene silencing and tumour regression without obvious toxicity observed in animal trials. The presentation will include methods for large-scale EVs purification with reservation of native shape and original function. Size, zeta potential, and electronic microscopic morphology analysis reveal that this method eliminated impurity but also avoid of aggregation and particle disruption.

Poster Session S03 – EVs and Biofluids
Chairs: TBD 5:15–6:30 p.m.

PS03.01

Sweating the small stuff: extracellular vesicles from sweat

Prateek Singh and Seppo Vainio
University of Oulu

Please see OPT03.03

PS03.02

ExRNA Atlas resource for sharing extracellular RNA data and for analysing it in the context of exRNA pathway knowledge

William Thistlethwaite[1], Sai Subramanian[1], Neethu Shah[1], Andrew R. Jackson[1], Robert Kitchen[2], Joel Rozowsky[3], James Diao, Timur Galeev[4], Anders Riutta[5], Kristina Hanspers[5], Alex Pico[5], Roger P. Alexander[6], David Galas[6], Andrew Su[7], Matthew Roth[1], Mark Gerstein[8] and Aleksandar Milosavljevic[1]

[1]Molecular & Human Genetics, Baylor College of Medicine, TX, USA; [2]Exosome Diagnostics; [3]Department of Molecular Biophysics & Biochemistry, Yale University, CT, USA; [4]Molecular Biophysics and Biochemistry, Yale University, CT, USA; [5]The Gladstone Institutes; [6]Pacific Northwest Research Institute; [7]Department of Molecular and Experimental Medicine, The Scripps Research Institute; [8]Molecular Biophysics and Biochemistry: Genomics, Genetics, and Bioinformatics

Introduction: The Extracellular RNA Communication Consortium (ERCC) has created the exRNA Atlas, a public web-accessible resource that includes over 1500 uniformly processed and curated exRNA profiles, on-line tools and links to computable knowledge resources. The exRNA Atlas can be accessed either directly (www.exrna-atlas.org) or via the Quick Links section in the exRNA Portal (www.exrna.org).
Methods: Datasets stored in the Atlas are derived from either RNA-seq or qPCR assays. All RNA-seq data are uniformly processed using the extracellular RNA processing toolkit (exceRpt), which generates sample-level quality control metrics, produces abundance estimates for small RNA species, and provides detailed alignment information for visualisation and validation. The Atlas also stores metadata for all samples using the GenboreeKB exRNA Metadata Tracker, a MongoDB-backed database service.
Results: Users may currently explore the 1567 exRNA profiles from 12 studies that are already present in the Atlas. We have integrated BioGPS to allow browsing of specific exRNA profiles within individual studies or across studies that employ similar experimental protocols. We have also created a census of miRNAs in the Atlas across body fluids and RNA isolation kits. User-supplied profiles may be compared across other profiles in the Atlas using an integrated PCA tool. The comparison may increase rigour and reproducibility by validating biofluid type and assay performance, it may also reveal novel associations and clustering patterns due to biological or experimental reasons. Finally, researchers can leverage our integrated network analysis tools including the Target Interaction Finder and Pathway Finder to interpret modules of covarying miRNA and other non-coding RNA species in the context of a growing exRNA pathway section in WikiPathways
Conclusion: The exRNA Atlas resource provides FAIR (Findable, Accessible, Interoperable, Reusable) exRNA profiling data, relevant tools, and computable exRNA pathway knowledge, thus catalysing data-intensive exRNA research.

PS03.03

How anticoagulation of plasma really affects EV yield and their properties?

Maarit Takatalo[1], Mari Palviainen[2], Sami Valkonen[3], Saara Laitinen[4] and Pia R-M. Siljander[5]

[1]Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy; [2]EV-core, Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy and Institute of Molecular Medicine Finland FIMM, University of Helsinki, Finland; [3]Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland, Finnish Red Cross Blood Service, Helsinki, Finland; [4]Finnish Red Cross Blood Service, Helsinki, Finland; [5]Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

Introduction: Extracellular vesicles (EVs) provide a common source material for biomarker studies or may in the future be used as biomarkers themselves. As a liquid biopsy, plasma or serum are relatively non-invasive providing a material rich in EVs from a variety of cells/tissues. Despite EVs' popularity, the issues of pre-analytical variables regarding plasma/serum as an EV source have not yet been explored in depth: several beliefs exist in the EV field, but data is lacking. In this study, we addressed the effect of anticoagulation to the yield and characteristics of the obtained EVs.
Methods: Pools of EDTA-, citrate-, acidic citrated dextrose (ACD)-anticoagulated plasma or serum were prepared from 10 healthy volunteers with identical centrifugation parameters and processed within an hour from blood collection. Plasma/serum was investigated for the presence (Coulter) and source of remnant cells (LSRII flow cytometer). EVs were isolated by ultracentrifugation and analysed for total protein and particle number (NTA) and by flow cytometry (Apogee). Western blotting was used to detect EV markers and ApoB.
Results: As expected, the largest differences were found between the serum-derived and the three plasma-derived EVs. Differences in size profiles, the presence of platelet-derived EVs and lipid labelling were detected. However, the EV yield from serum was the same as from plasma. In contrast, differences in the EVs isolated from the three plasma sources showed only minute differences regarding the presence of platelet-derived EVs and total protein, but had some clear distinctions in other parameters.
Conclusion: Compared to the EVs from serum, the choice of anticoagulation may not be the most crucial factor determining EV-yield or quality when pre-analytical conditions are considered. However, since the EVs had some unique characteristics depending on the source plasma, the choice of anticoagulant may be relevant in regard of some downstream analyses.

PS03.04

Serum miRNAs level is affected by feeding bovine and porcine milk in newborn piglets

Delin Lin, Liyuan Yao, Qianyun Xi, Jiajie Sun, Ting Chen and Yong-Liang Zhang

College of Animal Science, South China Agricultural University, Guangdong, China

Breast milk is the first and most important nutrient source for mammals after birth. Studies have indicated that 12 kinds of body fluid contain miRNAs, and milk has the highest concentration of total RNA. In milk, miRNA may be encapsulated in exosome, or bind to lipoprotein. In the present study, we hypothesised that miRNAs in breast milk can be absorbed by newborn piglets. Firstly, qRT-PCR was used to compare miRNA level between porcine and bovine milk, and four miRNAs (miR-2284x, miR-2291, miR-7134 and miR-1343) were identified with significant difference. Secondly, *in vivo* test was conducted to compare miRNA level in piglet serum after feeding porcine or bovine milk. Twelve piglets

were randomly selected from four sow's litters, 4 form each litter, and fed with bovine milk, while 12 other piglets took milk from their own mother. Serum samples of each piglet were collected on day 1, 3, 7 and 14, respectively. Total RNA was extracted from serum by Trizol method, and qRT-PCR was used to detect serum miRNA level quantitively. We confirmed that four miRNAs, miR-2284x, miR-2291, miR-7134 and miR-1343, showed significantly different levels in newborn piglets' serum after taking different kinds of milk. Meanwhile, porcine small intestinal epithelial cells (IPEC-J2) was co-cultured with bovine and porcine milk exosome and their exosome-free whey, respectively. The relative levels of four miRNAs in different experimental groups were detected to be higher than those in control group. The results indicate that not only miRNAs in milk exosome but also in exosome-freee whey can be absorbed by IPEC-J2 cells. Our findings suggest that miRNAs in breast milk can be absorbed by piglets via digestive tract. More importantly, different kinds of milk cause differences in miRNA absorption and levels in infant serum.

PS03.05

Differentially expressed exosome miRNAs induced by blood flow restricted exercise – possible effectors of endogenous organ protection and muscle hypertrophy

Jesper Just[1], Mette Sloth[1], Yan Yan[2], Kristian Vissing[3], Jørgen Kjems[2] and Kim Ryun Drasbek[1]

[1]Department of Clinical Medicine, Centre of Functionally Integrative Neuroscience, Aarhus University, Aarhus, Denmark; [2]Interdisciplinary Nanoscience Centre, Aarhus University, Aarhus, Denmark; [3]Section of Sport Science, Department of Public Health, Aarhus University, Aarhus, Denmark

Introduction: Remote ischemic conditioning strategies delivers an expanded potential as activation of endogenous organ protection during prolonged ischemia, and have shown promising results as additional acute treatment for myocardial infarct and stroke. However, at-risk subjects or patients with chronic conditions might also benefit from a prophylactic conditioning regiment. Here, blood flow restricted exercise (BFRE) is of special interest. BFRE is initiated by applying external pressure to the proximal part of the lower or upper extremities, which occludes venous outflow flow but maintains arterial inflow to the extremity. Combining BFRE with low-intensity training have demonstrated the ability of this method to increase muscle strength and hypertrophy. However, BFRE may also activate the endogenous organ protection seen in acute conditioning strategies, as similar biological pathways could be involved. A possible effector of ischemic conditioning is blood-borne micro RNAs (miRNA) carried in small extracellular vesicles (EVs). These released encapsulated miRNAs have the potential to change cellular protein expression both locally and systemically.
Methods: To investigate which known or novel miRNAs were up- or downregulated during BFRE, small EV RNAs (<50 bp) were isolated from plasma of five healthy human subjects pre and post BFRE. The isolated RNAs were sequenced by NGS and differential expression analysis was carried out using the Deseq2 software package in R.
Results: We show that several known miRNAs were up- and downregulated following BFRE. These miRNAs were compared to the existing literature and some of them showed interesting associations, suggesting a protective effect in ischemic disease.
Conclusion: Further investigations of these miRNAs might help to rebuild the beneficial underlying molecular mechanisms of ischemic conditioning and BFRE, and could offer new therapeutic targets in pathologies involving damaging hypoxia.

PS03.06

Minimal volume of urine for microvesicles detection

Luca Musante[1], Sai Vineela Bontha[2], Christine Rudy[1], Joanne Lannigan[3], Valeria Mas[4] and Uta Erdbruegger[1]

[1]Department of Medicine/Nephrology Division, University of Virginia, VA, USA; [2]Translational Genomics Transplant Laboratory, Division of Transplant, Department of Surgery, University of Virginia, VA, USA; [3]School of Medicine, Flow Cytometry Core, University of Virginia, VA, USA; [4]Translational Genomics Transplant Laboratory, Division of Transplant, Department of Surgery, University of Virginia, VA, USA

Introduction: Urinary extracellular vesicles (UEVs) provide a relative novel source of valuable biomarkers for kidney and urogenital diseases. As a matter of fact, so far the bulk of the research has focused mainly on exosomes as the primary source of extracellular vesicles (EVs). Only recently, have urinary microvesicles/microparticles been regarded as an additional important fraction of EVs carrying biomarkers. The number of MVs released by podocyte has shown to be higher in the urine of patient with diabetes mellitus type 1 without any kidney complications for instance. This study aims to investigate what is the minimal amount of urine which enables the detection and characterisation of MVs.
Methods: First morning void urine was centrifuged at relative centrifugation force RCF of 3200*g*. The supernatant was split in 0.5, 1.0, 1.5, 3.0, 4.5, 9.0 and 13.5 ml fractions to enrich MVs by centrifugation at RCF of 20,000*g*. Tunable resistive pulse sensing, imaging flow cytometry, cryo-transmission electron microscopy (TEM) and extraction of RNA were the techniques adopted to establish the minimal volume of urine to provide material for analysis. RNA was isolated from the MV pellet of 0.5 ml urine fraction for miRNA analysis.
Results: MVs could be detected by TRSP, and imaging flow cytometry and, it was possible to extract RNA and identify miRNA (that are previously identified in cell free healthy urine) using qPCR, already starting from 0.5 ml of urine with an estimated 1.5×10^8 particles in TRSP. Cryo-TEM provided adequately good images starting from a minimal volume of 1.5 ml of urine with MVs of the size which corresponded to the particle size distribution established in TRSP. However smaller vesicles with a diameter ≤100 nm were also detectable.
Conclusion: Depending on the sensitivity of the technique in use, a minimal volume of 0.5 ml urine can be useful for particle enumeration, MVs surface phenotyping and RNA analysis.

PS03.07

The phenotypical changes of plasma EVs over time in healthy donors

Rikke Baek[1], Morten Hjuler Nielsen[2], Jaco Botha[2], Lotte H. Pugholm[1], Evo K. L. Soendergaard[1], Kim Varming[1], Aase Handberg[2] and Malene M. Jorgensen[1]

[1]Department of Clinical Immunology, Aalborg University Hospital, Aalborg, Denmark; [2]Department of Clinical Biochemistry, Aalborg University Hospital, Aalborg, Denmark

Introduction: Extracellular vesicles (EVs) in plasma have a great diagnostic potential as biomarkers for several diseases. In order to use EVs in a clinical setting, it is of great importance to know whether the protein phenotypes of EVs in a healthy cohort changes over time. In this study, we collected blood from 10 apparently healthy donors over a period of 6 weeks to determine the long-term (week-to-week) as well as the short-term (day-to-day) variance of EV concentration and composition. Furthermore, blood cell counts were determined.
Methods: Venous peripheral blood (EDTA and CPDA) was obtained from 10 healthy donors once a week over a period of 6 weeks. Furthermore, blood samples were drawn from five of the donors daily during one week. Blood cell counts were measured by a Sysmex XN-1000. Small EV concentration and composition were analysed by the EV Array (1) using 29 selected surface-markers. The antibodies used to capture the EVs included antibodies against EVs in general (CD9, CD63, CD81, Alix, Flotilin-1 etc.), and immunological and inflammatory markers (CD4, CD8, CD80, HLA ABC, HLA DR/DP/DQ, TNF RI and RII etc.). Flow cytometry was used to analyse the larger vesicles (0.1–1.0 μm) for their content of phosphatidylserine, CD41 and CD36.
Results: In total, 80 plasma samples were collected and analysed. Large inter-individual variation was found in both cells and EVs. The long-term intra-individual variation in blood cells varied for some of the cell types significantly over time, which was not seen in the contents of small EVs. Smaller short-term and intra-individual variation (day-to-day) variation were observed in the cellular composition, but this was not reflected in the obtained phenotypes of EVs.
Conclusion: A few of the selected surface markers of the EVs showed minor changes over time, although this did not reflect the significant

changes identified on the cellular level. Hence, EVs tend to be a stable diagnostic biomarker source.

Reference

1. Jorgensen M et al., *J Extracell Vesicles.* 2013; 2: 3402/jev.v2i0.20920 2013.

PS03.08

Purification, molecular characterisation and initial functional characterisation of the EVs derived from renal cell carcinoma (RCC) and human sweat

Geneviève Bart, Anatoliy Samoylenko, Khem Giri, Fabienne Wagner, Hanna Thoma, Prateek Singh and Seppo Vainio

University of Oulu, Finland

Introduction: The extracellular vesicles (EVs) secreted by cells into the body fluids serve to remove cellular waste products but they also act as molecular transport vehicles to nearby or distant cells. While the detailed *in vivo* function of secreted EVs remains still poorly characterised identification of their cargo content may serve to provide valuable information about the status of cells with putatively great value as novel diagnostic markers besides other important medical potentials.
Methods: Two types of EVs were used in this study: EVs secreted by mouse renal carcinoma cells in normal or hypoxic conditions were collected from conditioned media and body fluid EVs were purified from human sweat. Purified RNA was sequenced with Iontorrent PGM (small RNA) and RNA annotation was done using Genboree Workbench. EV DNA was sequenced with NextSeq550 (Illumina) using whole genome approach. We also studied the effect of such EVs on mobility and proliferation of the recipient cells. To functionally test the EVs we applied them to mouse, dog and human derived cells lines and studied putative changes in gene expression based on the most abundant miRNA identified in our EVs.
Results: The sequencing data revealed that a large number of the RNA species that associated with EVs, were non-coding RNAs such as rRNA, tRNA, miRNA, lncRNa but also anti-sense ones. Fragments of mRNA were detected as well. Interestingly the EV associated DNA sequences depicted relatively widely distributed but chromosomally restricted "hot spot" segments including the mitochondria. In the functional assays the EVs had a notable impact on cell proliferation, cell motility and cell survival and lead to changes in mRNA expression in line with the presence of miRNAs in the EVs.
Conclusion: Characterisation of nucleic acid cargo of the EVs secreted by the two model systems, renal cell carcinoma (RCC) and sweat identifies a wealth of RNA and DNA sequences with diagnostic potential. We can conclude that detailed functional tests need to be carried out to classify the regulatory roles of the EVs, preferably eventually at the single EV and cell level.

PS03.09

Characterisation of extracellular vesicles released from adult mouse retina

Jason Mighty[1], Jing Zhou[1], Alberto Benito-Martin[2] and Stephen Redenti[1]

[1]CUNY Lehman College, NY, USA; [2]Weill Cornell Medical College, NY, USA

Extracellular vesicles (EVs), are lipid enclosed cell fragments with diameters ranging from 50 nM to 2 mM and released from most cell types including embryonic stem cells, hematopoietic stem cells and neurons. EVs have been shown to be involved in cell-cell communication via transfer of protein, mRNA and miRNA. EVs encapsulate factors representative of cell of origin genotype and phenotype. Currently there is limited research on EVs released from retinal tissue. In this study, we analysed the release rate, concentration and content of EVs released from adult mouse retina *in vitro*. EV release rate and ultrastructure were analysed using SEM and Nanosight analysis. Under standard culture conditions mouse retinal tissues were shown to release 1041.67 vesicles/hour with an average diameter of 196 nm. Using immulogold TEM and western blotting mouse retina derived EVs were shown to incorporate the tetraspanin proteins, Tsg101 and CD63. Genetic analysis revealed the presence of mRNA species within EVs comparable to those present in adult retinal tissue. These findings demonstrate that the adult mouse retina releases EVs and Future work will reveal potential influence of EVs in adult retinal function.

PS03.10

The exRNA virtual biorepository: a biospecimen catalogue service for sharing biofluid and tissue samples

Aleksandar Milosavljevic[1], Sai Subramanian[1], William Thistlethwaite[1], Andrew R. Jackson[1], Neethu Shah[1], Sameer Paithankar[1], Matthew Roth[1], Bob S. Carter[2], Fred Hochberg[3], Matt Huentelman[4], Kendall Jensen[4], Jorge Arango[5], Yashar Kalani[6], Julie Saugstad[7], Theresa Lusardi[8], Joseph Quinn[0] and John Nolan[10]

[1]Molecular & Human Genetics, Baylor College of Medicine; [2]Center for Theoretical and Applied Neuro-Oncology, University of California, San Diego, CA, USA; [3]Neurosurgery, University of California, San Diego, CA, USA; [4]Neurogenomics Division, Translational Genomics Research Institute; [5]Neurosurgery, Barrow Neurological Institute; [6]Department of Neurosurgery, University of Utah School of Medicine, UT, USA; [7]Anesthesiology and Perioperative Medicine, Oregon Health and Science University, OR, USA; [8]Computational Biology, Oregon Health and Science University, OR, USA; [9]Neurology, OHSU School of Medicine; [10]The Scintillon Institute, CA, USA

Introduction: The exRNA virtual biorepository (EVB) is a cloud-hosted"virtual" repository of biospecimens developed by the Extracellular RNA Communication Consortium (ERCC) for sample tracking, discovery, and sharing within the scientific community. The EVB supports the sharing of information about both biofluid- and tissue-derived samples that reside at participating laboratories and institutions. There are currently 4800+ biofluid samples in the EVB.
Methods: The EVB follows a spoke-hub model, where the hub functions as a central entity that receives updates regarding available biosamples at participating institutions and provides access to the combined information to researchers. All data and services are exposed via a FAIR REST API.
Results: The EVB Hub's portal page allows faceted filtering of samples by various data elements such as biofluid type, diagnosis at the time of sampling, and donor demographics. Searches can be saved for future use. When viewing search results, users can review additional details about the samples, decide whether to include them in an order cart, and then place the order. The hub breaks the cart into sub-orders for each participating institution, and aids in back-and-forth mediation between sample providers and sample requesters, so that requests are processed in a trackable, seamless, and timely manner. Participating institutions that provide samples and researchers requesting samples can be rated by the respective counter-party at the end of the transaction, similar to popular online marketplaces, and rating information is available as part of an institution's or user's profile to aid decision-making. We have implemented a model extension mechanism to support various research efforts – for example, the cerebrospinal fluid (CSF) consortium includes additional metadata about extracellular vesicle and RNA extraction methods, as well as vesicle quantification information from techniques such as NTA, TRPS, and vesicle flow cytometry.
Conclusion: The EVB Hub portal will be made accessible for public use in the coming months. In the meantime, the consortium anticipates adding several thousand samples to the current collection, thus catalysing exRNA research via sample sharing.

PS03.11

Yield, physicochemical properties and pharmacokinetics of exosomes derived from mouse cell lines

Chonlada Charoenviriyakul[1], Yuki Takahashi[2], Masaki Morishita[2], Akihiro Matsumoto[2], Makiya Nishikawa[2] and Yoshinobu Takakura[2]

[1]Kyoto University, Kyoto, Japan; [2]Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

Exosomes are small membrane vesicles secreted from cells and are expected to be used as drug delivery systems. Characteristics of exosomes, such as yield, physicochemical properties, and

pharmacokinetics, are important for the purpose and may be different among different cell types. However, there is limited information about the effect of cell type on these characteristics. In the current study, we evaluated these characteristics of exosomes derived from five different types of mouse cell lines: B16BL6 murine melanoma cells, C2C12 murine myoblast cells, NIH3T3 murine fibroblast cells, MAEC murine aortic endothelial cells, and RAW264.7 murine macrophage-like cells. A differential ultracentrifugation method was used to collect exosomes. The exosomes collected from all the cell types were negatively charged globular vesicles with a diameter of approximately 100 nm, and not statistically different. C2C12 and RAW264.7 cells produced more exosomes than the other types of cells. To evaluate the pharmacokinetics of the exosomes, they were labelled with a fusion protein of *Gaussia* luciferase (gLuc) and lactadherin (LA) by transfection of cells with gLuc-LA-expressing plasmid vectors. After intravenous injection into mice, all the gLuc-LA-labelled exosomes rapidly disappeared from the systemic circulation and mainly distributed to the liver. Fluorescent immunostaining revealed that the exosomes administered by intravenous injection were mainly taken up by F4/80^{+} macrophage sin the liver irrespective of the types of exosome-producing cells. In conclusion, these results indicate that the exosome yield was significantly different among the cell types, whereas the physicochemical properties and pharmacokinetics were comparable among all the exosomes examined.

PS03.12

Phospholipid influence prompts the need for an anti-TF antibody to specifically measure tissue factor activity on microvesicles

Loris Vallier[1], Tarik Bouriche[2], Amandine Bonifay[1], Jeremie Bez[2], Françoise Dignat-George[3], Romaric Lacroix[3] and Philippe Poncelet[2]

[1]Aix-Marseille Université, VRCM, UMR-S1076, INSERM, UFR de Pharmacie, Marseille, France; [2]Research and Technology Department, BioCytex, Marseille, France; [3]Aix-Marseille Université, VRCM, UMR-S1076, INSERM, UFR de Pharmacie, Marseille, France and Department of Haematology and Vascular Biology, CHU La Conception, APHM, Marseille, France

Introduction: Tumours and inflammatory blood cells express tissue factor (TF) and release TF-positive microvesicles (MV-TF) which activate blood coagulation. Many studies suggest that elevated MV-TF number or activity levels correlate with high risk of thrombosis. Several versions of a functional test measuring MP-TF activity via FXa generation have been described but their TF specificity and the influence of the phospholipid environment remain unclear. Our work aims to evaluate the influence of phospholipids on the TF activity of MVs.

Methods: Factor Xa generation assay was performed on TF+MVs from HAP-1 myeloïd cell line and vesicular phospholipids as TF-free MVs including (i) MVs from knocked-out TF (KO-TF) HAP-1, (ii) erythrocyte-derived MVs (ery-MVs) and (iii) platelet-derived MVs (PMVs). To measure TF-specific activity, the measure was carried out in parallel with an irrelevant antibody and a fully blocking anti-TF antibody (SBTF1, BioCytex).

Results: Factor Xa generation assay carried with various amounts of TF-free MVs showed a dose-related non-specific activity which is detectable from 2,5.10^{5} KO-TF HAP-1 or Ery-MVs or 2.10^{6} PMVs. For exemple, KO-TF HAP-1 MVs induce 220 ± 120 relative fluorescence units per minute (RFU/min) with 2.10^{6} MVs. Similarly, a residual activity (390 ± 40 RFU) which is not inhibited by a specific anti-TF antibody was measured using the same amount of TF+ HAP-1 MVs. Moreover, when excess amounts of KO-TF HAP-1 MVs, ery-MVs or PMVs were incubated with a fixed amount of TF+ MVs, a significant increase in factor Xa generation was observed which is not inhibited in presence of an anti-TF inhibitory antibody (respectively 149 ± 15%, 127 ± 20% and 134 ± 17% of initial activity with addition of 2.5 × 10^{5} TF-free MPs).

Conclusion: This study shows that the presence of vesicular phospholipids in the surrounding environnement significantly impact on the measurement of the MV-dependent FXa activity. This artefact requires the mandatory use of an inhibitory anti-TF antibody in the assay to measure only TF-specific FXa generation. Results from commercial assays not using such specific inhibition should be interpreted with caution.

Poster Session S04 – Isolation, Characterisation and Detection of EVs
Chairs: Nicole Noren Hooten and TBD 5:15–6:30 p.m.

PS04.01

Easy extracellular vesicle detection on a surface-functionalised power-free microchip

Ryo Ishihara[1], Tadaaki Nakajima[2], Asuka Katagiri[1], Yoshitaka Uchino[1], Kazuo Hosokawa[3], Mizuo Maeda[3], Yasuhiro Tomooka[2] and Akihiko Kikuchi[1]

[1]Department of Materials Science and Technology, Tokyo University of Science, Tokyo, Japan; [2]Department of Biological Science and Technology, Tokyo University of Science, Tokyo, Japan; [3]Bioengineering Laboratory, RIKEN

Please see OPT03.02

PS04.02

Methodological considerations for nanoparticle tracking analysis (NTA) of neat biofluids obtained from cardiac surgery

Andrew I.U. Shearn[1], Costanza Emanueli[2] and Giovanni Biglino[1]

[1]University of Bristol, United Kingdom; [2]Bristol Heart Institute, University of Bristol, United Kingdom

Introduction: Exosomes are potential biomarkers in the cardiac surgery setting. The use of NTA technology with neat biological samples and the way different parameters affect NTA results in this context have not been fully explored, particularly with the latest technology/software. This study sought to determine important parameters that need to be considered when analysing neat human biofluids with NTA.
Methods: Human plasma and pericardial fluid, collected from cardiac surgery patients under ethical approval, were analysed on an NS300 (Malvern, Malvern, UK) using NTA software v3.2 with a syringe pump. Calibration of the machine was performed using artificial exosomes (HansaBioMed, Tallin, Estonia).
Results: Calibration was performed successfully and recording reproducibility verified. Video length has a substantial impact on total particle concentration, the total number of particles counted in the 30–120 nm (exosomal) range being 42% higher when 150 s videos are used compared to 90 s. More videos reduce the variability of absolute particle count (SD 2.68×10^8 30–120 nm particles/mL for 2 videos vs. 2.02×10^8 for 4 videos). Unfiltered samples showed substantial underestimation of particles in the exosomal range due to reflections, whilst filtration of samples with a 0.22 μm filter prevented large aggregates interfering with the measurements. Reasonable variations in the max jump settings and small necessary focus adjustments do not substantially affect quantification of nanoparticles compared to default settings. Camera levels and detection thresholds should be kept consistent between different patients.
Conclusion: NTA can analyse nanovesicles in whole biofluids. Certain settings must be optimised prior to acquisition, particularly video length and the number of videos. In addition to optimising focusing of the instrument and ensuring thorough cleaning between samples, sample filtration eliminates larger particles that can interfere with processing and mask smaller particles.

PS04.03

An affinity-based method for efficient recovery of tumour-derived evs from conditioned media and human plasma that can be used for detection of actionable mutations in liquid biopsy applications

Catherine Taylor[1], Sheena Fry[1], Anirban Ghosh[2], Jeremy Roy[1], Nicolas Crapoulet[3], Simi Chacko[1], Annie-pier Beauregard[1], Sebastien Fournier[1], Biji Anish[1], Ian C. Chute[1], Remi Richard[1], Stephen M. Lewis[2] and Rodney J. Ouellette[2]

[1]Atlantic Cancer Research Institute, New Brunswick, Canada; [2]Department of Chemistry and Biochemistry, Université de Moncton, New Brunswick, Canada; [3]Department of Chemistry and Biochemistry, Faculty of Medicine, Université de Sherbrooke, New Brunswick, Canada

Introduction: Circulating DNA in blood is becoming an increasingly important resource for detection of "actionable" mutations that are important for determining therapeutic strategies in the treatment of cancer patients. In addition to cell-free DNA (cfDNA) and circulating tumour cell DNA, extracellular vesicles (EVs) are gaining recognition as an important source of tumour-derived DNA in liquid biopsy applications. Vn96 is a synthetic peptide with an affinity for heat-shock proteins that has been developed into a fast and efficient method for EV isolation from a variety of biofluids. In this study, Vn96 peptide was used to isolate tumour EV-derived DNA in order to assess the detection of actionable mutations from both EV-spiked plasma and breast cancer patient plasma samples.
Methods: Nanoparticle tracking analysis (NTA) was used to quantify the number of EVs isolated using increasing concentrations of Vn96 from conditioned cell culture media and from normal human plasma spiked with purified PANC10.05 (*KRAS G12D* heterozygous pancreatic cell line) EVs. Recovery of PANC10.05 EV DNA from spiked plasma was assessed by digital drop PCR analysis of *KRAS* (WT and G12D). DNA was isolated using Vn96 from 1 mL samples of breast cancer patient plasma and actionable mutations were detected by next-generation sequencing. A comparison to cfDNA isolated from the same plasma samples was made. Since the number of EVs in blood may be an important diagnostic marker of disease in its own right, NTA was used to explore correlations between the number particles per mL in breast cancer patient plasma (isolated using Vn96) and disease stage.
Results: A good correlation (r = 0.95) was observed between Vn96 binding studies using conditioned media from two different cell lines, with an average isolation of 5.7×10^8 particles per μg Vn96. Vn96 was also found to be able to efficiently recover EVs from plasma, with >80% recovery of EV-derived DNA from spiked plasma samples. Sufficient DNA for next-generation sequencing was obtained from only 1 mL of plasma from patients with advanced breast cancer.
Conclusion: Affinity purification of EVs using Vn96 peptide provides a simple, scalable technique that can be used for EV research and which has the potential to be useful in the development of liquid biopsy technologies for clinical diagnostics.

PS04.04

Evaluation of individual exosomes down to 10 nm in microfluidic devices

Takanori Ichiki and Takanori Akagi

University of Tokyo, Japan

Currently, one can use various methods for characterising a heterogeneous population of extracellular vesicles (EVs), e.g. transmission electron microscopy (TEM), atomic force microscopy (AFM), nanoparticle tracking analysis (NTA), flow cytometry and so forth. Besides them, authors have recently developed a microfluidic-based analytical platform that enables the multiparametric characterisation of nanovesicles by concentration, diameter, zeta potential, and surface antigenicity (1). Unfortunately, however, most of the above methods are suffering from difficulty in detecting small vesicles below 50 nm with the exception of TEM, and there is a strong demand for extending the detection size limit to clarify the whole picture of EVs including exosomes. In this presentation, we will report the successful improvement of detecting individual EVs down to 10 nm on our analytical platform.
As a demonstration of the improved performance, size measurement of EVs was conducted as follows. After cultivation with a serum-free medium for 48 h, culture supernatants of human breast cancer SkBr3 and leukaemia HL60 cells were centrifuged at 300*g* for 10 min, at 2000*g* for 20 min and at 10,000*g* for 100 min. The clarified supernatant, used as a feed sample, was further centrifuged at 100,000*g* for 200 min. Vesicles in resulting supernatant (100ksup) and pellet (100kpt) were evaluated. Size ranges of SkBr3's

EV in the feed, 100ksup and 100kpt were 18.7–204, 21.5–136 and 5.1–104 nm, respectively, while those of HL60's EV in the feed, 100ksup and 100kpt were 34.1–287, 8.7–271 and 8.6–152 nm, respectively. In the case of SkBr3, ratios of vesicles of 50 nm or less to the whole were 5.6%, 19.0% and 59.4% for the feed, 100ksup and 100kpt, respectively. And, in the case of HL60, ratios of vesicles of 50 nm or less to the whole in the feed, 100ksup and 100kpt were 39.6%, 60.0% and 92.2%, respectively. Thus the improvement in detection limit down to 10 nm can shine a spotlight on innegligible amount of subjects that could not be measured until now.

Reference

1. Akagi T et al., *PLoS One*. 2015; 10: e0123603.

PS04.05

High throughput qualitative and quantitative analysis of plasma-bound microvesicles

Wei Zhang[1], Neva Kandzija[1], Vuyane Mhlomi[2], Ana-Sofia Cerdeira[3], Gavin Collett[1], Alexandra Burdujan[1], Ian Sargent[1], Christopher Redman[1], David Ferguson[1] and Manu Vatish[1]

[1]Oxford University, Oxford, United Kingdom; [2]Oxford, United Kingdom; [3]NHS

The current go-to technique for microvesicle analysis utilises flow cytometers capable of single-event multi-parameter analysis. Using a conventional LSR-II and placental derived microvesicles as a model, we report a detailed method to (1) optimise flow cytometer scatter resolution and determine size limitation for microvesicle detection in an antigen and machine specific context; (2) incorporate a "dump channel" (used in rare event analysis) to enhance microvesicle detection specificity in complex biological fluids. Blood samples from women with normal term pregnancy were collected with informed consent. We found significant decreases in the number of circulating trophoblast extracellular vesicles (event positive for placental alkaline phosphate) in 100ul of the same donor's platelet-free plasma compared to platelet-poor plasma for both uterine vein (670.8 ± 371 vs. 2781 ± 1534, $n = 6$, $p = 0.03$) and peripheral samples (281.3 ± 113.3 vs. 522.5 ± 145.9, $n = 8$, $p = 0.008$). The natural gradient of trophoblast extracellular vesicles seen in uterine vein compared to paired peripheral blood was only visible when using platelet-poor plasma ($p = 0.002$) and was not visible when using platelet-free plasma (the generally recommended material for plasma microvesicle analysis). Our data presents an optimised technique for microvesicle detection and also cautions against use of platelet-free plasma since significant signals may be lost.

PS04.06

Nanoarray platform for high-throughput single exosome proteomic characterisation

Philippe DeCorwin-Martin, Eun Hae Oh, Rosalie Martel and David Juncker

McGill University and Genome Quebec Innovation Centre, Montreal, Canada

Introduction: Extracellular vesicles (EVs) are highly heterogeneous in their composition, and there is a need to characterise subpopulations of EVs that may be key in understanding the effects and mechanisms by which they shape cellular processes. Whereas electron microscopy identifies single EV, the throughput is too low, yet most other methods only provide averaged data. Recently significant progress has been achieved by flow cytometry for high throughput analysis of single EVs. Here, we propose a nanoarray platform to characterise single exosomes immobilised on a surface in a high-throughput manner and help differentiate exosome subpopulations.
Methods: A nanoarray of anti-mouse IgG was printed onto a glass slide using lift-off nanocontact printing, and the surface was passivated before incubating with mouse monoclonal capture antibodies. The nanoarray consists of 100 nm spots that capture single exosomes by size exclusion. They are separated by a 2 mm pitch such that adjacent captured vesicles can be easily distinguished. Exosome samples, purified from cell supernatant using ultracentrifugation or size exclusion columns, are incubated on the nanoarray overnight and detected using a fluorescently tagged detection antibody. The slide is then imaged on a fluorescent microscope, allowing for up to four fluorescent channels.
Results: Single vesicle capture was demonstrated on the nanoarray in proof-of-principle experiments using fluorescently labelled liposomes. Vesicles containing down to a few fluorophores could be detected over the background.
Conclusion: The heterogeneity of extracellular vesicles calls for methods that can measure single vesicles to allow for an accurate description of vesicle composition. With the nanoarray's ability to capture single exosomes in a high-throughput method and detect up to four different co-expressed proteins, vesicle subpopulations can now be studied for their distinct effects in cell processes.

PS04.07

Improved resolution in extracellular vesicle populations using 405 nm instead of 488 nm wavelength side scatter

Mark John McVey[1], Chris Spring[2], Vera A. Tang[3], Marc-Andre Langlois[3] and Wolfgang Kuebler[4]

[1]University of Toronto, Canada; [2]Keenan Research Centre (St-Michael's Hospital) Toronto, Canada; [3]University of Ottawa, Canada; [4]Charite University, University of Toronto, Canada

Introduction: Improvements in identification and functional assessments of extracellular vesicles (EVs) has led to a recent surge in EV publications investigating their roles as biomarkers and effectors of disease and repair. As this area of research evolves, a common limitation to allow meaningful comparisons is accurate and reproducible enumeration and characterisation of EVs in biological fluids. High sensitivity flow cytometry (FCM) is a popular strategy to assess EVs, however an impediment in using FCM is differing and often limited ability to resolve smaller EVs.
Methods: To address this limitation we propose the use of lasers with 405 nm (violet) in place of 488 nm (blue) side scatter (SSC) detection to obtain greater resolution of smaller EVs using high sensitivity FCM. To test this hypothesis we resolved latex and silicone reference beads as well as biological EVs from plasma and bronchoalveolar lavage fluid (BAL) using either violet or blue wavelength SSC EV detection and quantified the change in particle resolution.
Results: Mie scatter modelling reveals violet SSC improves resolution of small (100–500 nm) spherical particles with refractive indices (1.34–1.46) similar to EVs by approximately 2 fold in terms of light intensity and a nearly 20% increase in side scatter signal quantum efficiency. Reference beads showed improved resolution when detected by violet instead of blue SSC with nearly twofold decreases in coefficients of variation for 300–500 nm particles, and fivefold for 180–240 nm particles. Similar effects were seen when resolving EVs from plasma and BAL using both SSC wavelengths. Specifically, violet SSC detection allowed for greater sampling of smaller EVs, which is of particular relevance considering nanotracker analysis revealed in both plasma and BAL that most EVs were <300 nm.
Conclusion: Violet instead of blue SSC detection for high sensitivity FCM allows significantly greater resolution of EVs in plasma and BAL. The advantages of violet detection were exaggerated for smaller particles, hence these insights may prove especially helpful in detection of smaller EVs. Notably, this simple strategy is readily accessible and inexpensive for machines equipped with 405 nm SSC or the ability to accommodate appropriately positioned 405/10 nm bandpass filters in their detection arrays.

PS04.08

Best before – lyophilisation as novel storage alternative for extracellular vesicles

Julia Frank and Gregor Fuhrmann

Helmholtz-Institute for Pharmaceutical Research

Introduction: Extracellular vesicles (EVs) are increasingly studied for biosignalling, pathogenesis and biomedical applications (1). Currently, the international consensus supports their storage at −80°C (2). Lyophilisation (freeze-dry) of EV would allow easy handling at room temperature (RT) and thus significantly boost their expanded investigation. However, EV behaviour upon lyophilisation remains largely unknown. We comprehensively evaluated for the first time the freeze-

drying impact on various EV's stability and functionality upon model enzyme loading, and we assessed the effect of cryoprotectants.
Methods: EVs were isolated from 48 h conditioned culture medium by ultracentrifugation (120,000*g*, 2 h), loaded with glucuronidase *via* saponin treatment (3) and purified by gel filtration (Sepharose CL-2B). EVs were stored at RT, 4 or −80°C, and lyophilised with/without addition of mannitol or trehalose, and analysed by nanoparticle tracking analysis and electron microscopy (TEM, phosphotungstic acid stain). Residual enzyme activity was assessed with fluorescein glucuronide (37°C) and compared to liposomes (phosphocholine/cholesterol 60:40 mol%).
Results: After 14 d EV from A549 lung cancer, MSC stem or HUVEC endothelial cells and liposomes showed average sizes of ~190 nm for all storage conditions. A 60% decrease in particle number was observed for A549 EV during freeze-drying compared to storage at −80°C but was less pronounced for MSC (30%) and HUVEC EV (20%). Addition of 1 wt% mannitol caused cryoprotection and inverted this effect, with EV morphology not altered as imaged by TEM. The glucuronidase activity of loaded MSC EV was lost after 14 d of storage but addition of 4 *wt*% trehalose induced 80% recovery of enzymatic cleavage comparable to activity levels of liposomes (70% recovery), indicating that low sugar concentrations preserve the EV's functionality.
Conclusion: For the first time, we show that EV have natural stability during freeze-drying, further optimised by the addition of cryoprotecting sugars. Our findings provide new insight and a firm basis for exploring lyophilisation as novel EV storage modality.

References

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PS04.09

EV core – the world's first technology platform dedicated to extracellular vesicle isolation and analytics

Maija Puhka[1], Mari Palviainen[2] and Pia R-M. Siljander[3]

[1]Institute for Molecular Medicine Finland FIMM, University of Helsinki, Finland; [2]EV-core, Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy and Institute of Molecular Medicine Finland FIMM, University of Helsinki, Finland; [3]Division of Biochemistry and Biotechnology, Department of Biosciences/Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

Introduction: Extracellular vesicles (EVs) are rich in blood and other biofluids and as cross-kingdom signalosomes they present both a novel research target but also ample possibilities for utilisation. Particularly due to their biomarker potential and the non-invasive availability, EVs are investigated for diagnostic and therapeutic applications. However, currently the unmet need for specific analysis instruments, lacking standardisation and the need for better optimisation in the purification and basic characterisation of EVs hinders the progress.
Methods: As a novel infrastructure, we have set up an academic, non-profit EV technology core facility in the University of Helsinki. We offer an easier access to the state-of-the-art and emerging technologies for research groups, hospitals and authorities interested in EVs. The expertise of the EV core encompasses: (1) sample handling and storage, material requirements (plasma, urine, culture media, etc.); (2) EV isolation with ultracentrifugation, chromatography and kits; (3) low-amount methods: particle size and number, protein, nucleic acid, lipid, metabolite and EM analyses; (4) EV flow cytometry; (5) EV-specific data analysis/normalisation.
Results: During this first year, we have produced several SOPs for EV analyses and two new methodologies to improve EV isolation (patent investigation ongoing) and one method for purity analysis. We have participated in the development of biological EV reference materials. Through our clients, we are involved in research projects to which we contribute various analytical services. We are also addressing several basic questions from kit comparisons to pre-analytical considerations for EV isolation (see abstract on plasma vs serum EVs).
Conclusion: While the interest and applications for EV use are exponentially increasing, there is an unmet need to improve and standardise the basic isolation and characterisation methods and to develop reference materials to enable intra- and inter-laboratory comparison of results. Based on our experience so far, there is also an unmet need for such a core facility helping both newcomers to EV field and the more experienced in need of the specialised EV analyses. An academic EV core dedicated to these issues can significantly facilitate these important goals.

PS04.10

Large extracellular vesicles dominate the results of immunosorbent assays

Elmar Gool[1], Rienk Nieuwland[2] and Frank A. W. Coumans[1]

[1]Biomedical Engineering & Physics, Academisch Medisch Centrum; [2]Clinical Chemistry Department, Academisch Medisch Centrum

Introduction: Immunosorbent assays (ISA), such as the enzyme linked immunosorbent assay (ELISA), are widely used to phenotype extracellular vesicles (EVs). However, EV samples are heterogeneous and it is unknown to which extent ISA results reflect the antigen exposure of all EV present in a sample or of a subpopulation. Here we determine the effect of the EV diameter on the contribution to ISA results.
Methods: A diffusion model was developed to determine the diameter and amount of EV that are captured by an antibody-coated surface. The initial EV size distribution for the model was obtained from a conditioned cell culture supernatant, and the EV transport towards the surface was modelled with 1D particle diffusion described by the Stokes-Einstein relation. Subsequently, the contribution of the captured EV to the total number of epitopes was determined by assuming equal antigen surface density irrespective of the EV diameter.
Results: Small EV, arbitrarily defined as 50–200 nm, outnumbered large EV (400–1000 nm) by 10-fold in the initial sample. The model determined that this ratio will increase to 26-fold for captured EV on the antibody-coated surface. Since small EV diffuse faster than larger EV, small EV will travel longer distances to the surface. Consequently, relatively more small EV are captured than larger EV. However, because large EV have a larger surface area and contain up to 400-fold more epitopes, our model predicts that large and small EV will contribute to ~48% and ~28% of the total epitopes, respectively. The ratio of epitopes provided by small and large EV, that contribute to the ISA result, is thus 0.6.
Conclusion: This theoretical approach demonstrates that ISA results are influenced by the diameter of EV and mainly reflect the antigen exposure of an EV subpopulation. To validate this finding, we are currently performing verification experiments. In daily practice, our study indicates that ELISA signals are dominated by epitopes on large EV, whereas signals from label-free ISA methods will contain an askew contribution of small EV.

PS04.11

Characterisation of mycobacterial membrane vesicles

Vanessa Chang[1], Priscila Dauros-Singorenko[2], James Dalton[1], Cherie Blenkiron[1,2], Siouxsie Wiles[1], Simon Swift[1] and Anthony Phillips[2,3]

[1]Department of Molecular Medicine and Pathology, University of Auckland, Auckland, NZ; [2]School of Biological Sciences, University of Auckland, Auckland, NZ; [3]Department of Surgery, University of Auckland, Auckland, NZ

Introduction: Tuberculous and non-tuberculous Mycobacteria release membrane vesicles (MMVs), reported to range from 60 to 300 nm in diameter, predominantly contain lipoproteins and polar lipids. It is hypothesised that MVs facilitate delivery of virulence factors and function as "immune decoys" modulating host immune responses contributing to severe disease.
To better understand MMV biology we undertook the analysis of three species: *Mycobacterium smegmatis* (non-pathogenic, fast-grower), *M. abscessus* (human pathogen, fast-grower) and *M. marinum* (fish and opportunistic human pathogen, slow-grower). The *M. marinum*-

zebrafish model has been proposed to be one of the best models to study human tuberculosis.
Methods and Results: Different MMV parameters including composition, size, concentration and release with respect to cell growth and viability were studied. Nanoparticle tracking analysis and electron microscopy techniques were used to determine MMV concentration and size.
We isolated MMVs with mean diameters between 80–200 nm. SDS-PAGE protein profiles were similar for three isolations for each species with interspecies differences. DNA and RNA concentrations between 2–85 and 3–45 μg/ml of original culture respectively were obtained.
Conclusion: MMVs were produced throughout growth, with most produced at the transition between exponential and stationary phase. Stationary phase MMVs from *M. abscessus* were the largest (~200 nm) and contained more DNA than RNA (~20×) suggesting the existence of a selective packaging mechanism. MMVs from *M. smegmatis* and *M. marinum* contained equal levels of DNA and RNA. MMV production was correlated with cell viability using live/dead staining, showing that MMVs were produced by live cells suggesting vesicle production could be an active biological process. Purification of MMVs by density gradient centrifugation showed distinct MMV rich fractions in all species investigated, with different DNA and RNA patterns across the density layers suggesting heterogeneity among species. *In vitro* experiments challenging THP-1 cells with *M. marinum* vesicles showed that MMVs had a dose dependent effect on THP-1 cell viability. Further investigation is required to identify the active MMV components, the mechanism of killing and to characterise the effects of sub-lethal MMV challenges.

PS04.12

The use of fluorescent metabolites for the detection of exosomes from cancer cells

Alan M. Ezrin[1], Michael W. Graner[2] and Steven G. Griffiths[3]

[1]NX Development Corporation; [2]University of Colorado Denver, Anschutz Medical Campus, Dept of Neurosurgery, CO, USA; [3]X0S0ME

Introduction: Despite the labyrinthine paths taken to reach malignancy and the multiphenotypic character of individual cancers, all are affected by dysregulated metabolism. Initially glycolysis and anaplerotic pathways sustain biosynthetic requirements, mitochondrial activity innate or coopted are prequisites for tissue invasion and metastasis. *Ergo* beacons of altered metabolism are useful in cancer detection, location and treatment; a prime example is FDG PET.
Of particular importance in the tumorigenic metabolic landscape is the heme pathway: it promulgates carbon fixation, biosynthetic precursors, redox control and removal of toxic by-products. Glioblastoma management has been assisted by water soluble heme precursor 5-ALA (Gliolan): the drug is converted rapidly in cancer to a fluorescent bottleneck metabolite PPIX. The fluorescence enables complete surgical removal of brain cancer tissue since the metabolite binds to affected cell membranes. We wondered whether 5-ALA could be used to reveal evidence of hyperkinetic pathways from source cells or restored in EVs as markers for cancer detection and treatment.
Methods: EVs were harvested from oncosphere cultures of normal human astrocytoma and the glioblastoma F3-8, incubated with or without water soluble 5-ALA, by differential centrifugation. EVs were analysed for canonical proteins by western blot; PPIX presence was determined by NanoSight LM10 with a cut off filter at 565 nm to exclude non-specific particles.
Results: EVs were validated through the presence of CD63 and heat shock proteins among others. Analysis of a suspension of EVs by Nanosight enabled the identification of aggregates of brightly fluorescent particles using the 565 nM filter, indicative of origin or that 5-ALA metabolism can reflect dysregulated metabolism from source cells or *via* pathway(s) reestablished extracellularly.
Conclusion: The detection and management of cancer will depend on identification of key metabolic features by minimally invasive collection from blood or urine. In this study we demonstrate that it is possible to detect EVs from cancer cells with accelerated heme metabolism. Thus detection may be realised through administration of Gliolan to the patient prior to sample collection or mixture of purified EVs after collection.

PS04.13

Identification of a novel population of lipid-rich extracellular vesicles

Alanna Sedgwick[1], M. Olivia Balmert[1] and Crislyn D'Souza-Schorey[2]

[1]University of Notre Dame, IL, USA; [2]Department of Biological Sciences, University of Notre Dame, IL, USA

Extracellular vesicles (EVs) comprise a heterogeneous group of cargo-loaded vesicles, which are released from cells to mediate extracellular communication in normal physiology and disease. Such diversity in shed vesicles endows the cell with the ability to react to disparate physiological signals via the mobilisation of specific types of vesicles. The two best-characterised classes of EVs at present are exosomes and microvesicles, distinguished largely on the basis of size, but each identified with a unique set of cargo, size profile and mechanism of biogenesis. More recently, a relatively homogenous population of nanovesicles has also been described. Using a variety of fluorescence microscopy approaches and biochemical characterisation we have identified a novel population of large extracellular vesicles, which are largely devoid of classical markers used to distinguish the currently characterised EV populations. This new class of EVs encompasses lipid-rich extracellular vesicles (LEVs), and is formed at the cell surface in a wide range of cell types and requires the integrity of the microtubule cytoskeleton. LEVs exhibit unique morphological features and appear to function in lipid efflux. These vesicles represent an exciting avenue for future research particularly in diseases whose pathology may be responsive to lipid alterations.

LBP.44

Bioavailability of bovine milk-derived EVs for drug delivery application

Masaharu Somiya[1], Yusuke Yoshioka[2] and Takahiro Ochiya[2]

[1]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute; [2]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Japan

Introduction: EVs deliver their cargo to recipient cells and this ability can be utilized for the delivery system of therapeutic molecules. Some reports revealed that bovine milk is ideal raw material for the drug delivery application of EVs, since bovine milk is rich in EVs and widely available. However, toxicity and immunogenicity of bovine milk-derived EVs (mEVs) are not fully evaluated. In this study, we isolated mEVs and characterized its protein components. Furthermore, we determined the bioavailability of mEVs upon systemic administration into mice.
Methods: For the purification of mEVs, defatted bovine milk was treated with acetic acid to precipitate non-EV proteins, followed by ultracentrifugation. Protein components in mEV fraction were determined by western blotting, proteomic analysis, and ExoScreen method. Cellular uptake and cytotoxicity of mEVs were evaluated using mouse macrophage cell line Raw264.7. After the several intravenous administrations of mEVs into mice, toxicity, immunogenicity, and anaphylactic reaction were examined.
Results: Approximately 10 mg of EVs was isolated from one litter of bovine milk and mEV fraction contains typical EV marker proteins, such as tetraspanins and Rab family proteins. mEVs showed 120 nm in diameters and spherical shape. mEVs were efficiently taken up by Raw264.7 cells *in vitro* without affecting cell proliferation, suggesting that mEVs could be used for the delivery of therapeutic molecules. In the animal experiments, we did not observe any systemic toxicity upon intravenous administration. Some types of cytokines and chemokines in blood were slightly increased, however, anaphylactic reaction was not observed.
Summary/Conclusion: Taken together, mEVs are well-tolerated in the systemic administration and can be used as safe and cost-effective drug delivery system.

LBP.45

Recipient cell organelle separation for EV uptake studies: Tracking of extracellular vesicles

Ganesh Shelke[1] and Jan Lötvall[2]

[1]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Sweden; [2]Krefting Research Centre, University of Gothenburg, Sweden

Background: Extracellular vesicles (EVs) such as exosomes and microvesicle are known to delivery cargo like proteins, lipids, RNA, and DNA to the recipient cells. Transfer of EVs to recipient cells to deliver these cargos is essential to induce cellular phenotypic changes. Current methods to localize EVs in recipient cells are restricted to imaging of cells using co-localization of fluorescent probes. We propose a physical method that provides high-resolution separation of organelles that can be associated with EVs recipient cell trafficking.
Methods: EVs were isolated from mast cell line (HMC1.2) by differential centrifugation (16,500´g 20 min and 120,000´g 3 hr) followed by flotation on iodixanol gradient (182,300´g for 16 hours; SW40-Ti rotor). EVs were biotinylated by incubating it with EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) and free biotin was removed by dialysis (3.5 kDa filter) as per the manufacturer recommendations. Biotinylated-EVs were later incubated with HEK-293T cells for 60 min, after which cells were lysed (High salt, high pH buffer and sonication) to obtain crude organelles. Crude organelles carrying biotinylated EVs were further separated on iodixanol density gradient with two consecutive ultracentrifugation steps. Various iodixanol fractions were analyzed using immunoblotting for lysosomal (LAMP1) and endosomal protein (EEA1), as well as streptavidin-HRP based detection of EVs-biotin.
Results: High resolution separation of endosomal and lysosomal organelles fraction was obtained using this method. We found that biotinylated EV proteins were enriched in the endosomal fraction. A small quantity of biotinylated-EV proteins were also present in lysosomal enriched fraction.
Summary/Conclusion: Endosomal and lysosomal localization of EVs can be performed in recipient cell by iodixanol density gradient centrifugation. EVs were primarily enriched in the endosomal compartment, and only traces were detected in the endo-lysosomal compartment at the time point studied.

Poster Session S05 – EVs in Cardiovascular Disease
Chairs: TBD 5:15–6:30 p.m.

PS05.01

Proteomic profiling reveal Src as a novel microvesicle-associated biomarker for myocardial infarction

Olof Gidlöf[1], Mikael Evander[2], Thomas Laurell[1] and David Erlinge[3]

[1]Lund University; [2]Department of Biomedical Engineering, Lund University, Sweden; [3]Department of Cardiology, Clinical Sciences, Lund University, Sweden

Please see OPT02.05

PS05.02

Quantification of the circulating vesicle-bound pools of adipocytokines reveals that MFG-E8 and MIF are conveyed by plasmatic EVs

Maeva Durcin[1], Marine Malloci[2], Luisa Vergori[2], Severine Dubois[3], Gilles Simard[3], Olivier Hue[4], M. Carmen Martinez[2], Ramaroson Andriantsitohaina[2] and Soazig Le Lay[5]

[1]INSERM U1063/University of the French West Indies; [2]INSERM U1063; [3]INSERM U1063/Angers University Hospital; [4]University of the French West Indies; [5]INSERM

Introduction: Obesity-associated metabolic diseases are linked to dysregulated production of many factors secreted by adipose tissue, known as adipocytokines. Accumulating evidences suggest a role for circulating extracellular vesicles (EVs), significantly increased in obesity, in obesity-associated metabolic dysfunctions. Since EVs may convey hormones and metabolites, we aimed to evaluate their contribution in the secretion of adipocytokines.
Methods: EV subsets, including microvesicles (MV) and exosomes (EXO), were isolated from plasma samples collected from patients suffering of metabolic syndrome (MS) and quantified by NTA and flow cytometry. Patients were classified according to their body mass index (BMI): control (BMI < 27), overweight (27 < BMI < 30) and obese (BMI > 30). 22 adipocytokines circulating concentrations were successively measured on total, MV- and EV-depleted plasma samples by multiplex immunoassays.
We first showed that circulating MV and EXO populations were significantly increased with BMI supporting a role of these vesicles as metabolic relays in the context of obesity. Multiplex analysis of plasmatic adipocytokines confirms dysregulated production of these factors with increased BMI. Sequential depletion of MV and EXO from all plasma patients did not modify adipocytokine circulating levels, at the exception of MFG-E8 (Milk Fat Globule-EGF-Factor VIII) and MIF (macrophage migration inhibitory factor), which were decreased. Of interest, 37.3% of circulating MFG-E8 and 57.3% of circulating MIF were associated to EVs. Notably, MFGE-E8 preferentially associated with EXO (24%) whereas MV carried more than half of circulating MIF (50.6%). Nonetheless, EV-associated proportions of these two adipokines were unchanged with obesity suggesting that MFG-E8 and MIF constitutively used EVs as secretory pathways.
Results: Our results highlight that a significant proportion of MFG-E8 and MIF associate with EXO and MV, respectively, in plasma. Thus, this study emphasises the importance to consider EV secretory pathways in the metabolic actions of adipocytokines.
This study was approved by Angers University hospital ethical committee (NCT: 00997165) and received written consent from patients.

Funding: This work was funded by a research national grant (ANR MilkChEST n°ANR-12-BSV6-0013-04), by GIS APIS-GENE and the French Society of Diabetes.

PS05.03

Adipocyte extracellular vesicles increase leucocyte attachment to vascular endothelial cells

Rebecca M. Wadey[1], Katherine D. Connolly[1], Aled Rees[2] and Philip James[1]

[1]Cardiff Metropolitan University, Cardiff, United Kingdom; [2]Cardiff University, Cardiff, United Kingdom

Introduction: The hypoxic and chronic low grade inflammatory state of adipose tissue in obesity is associated with an overall increased risk of cardiovascular disease. The aim of this study was to determine if extracellular vesicles (EVs) derived from adipocytes cultured in a manner that mimics that of obese adipose tissue, play a functional role in the progression of cardiovascular disease, by promoting leucocyte attachment to vascular endothelial cells.
Methods: Differentiated 3T3-L1 adipocytes were incubated for 24 h in one of four conditions: "control" (serum-free medium (SFM), 95% air/5% CO_2), "TNFα" (SFM plus 30 ng/ml TNFα, 95% air/5% CO_2), "hypoxia" (SFM, 1% O_2) or "TNFα & hypoxia" (SFM plus 30 ng/ml TNFα, 1% O_2). EV were isolated from media by ultracentrifugation. Freshly isolated primary human umbilical vein endothelial cells (HUVEC) were treated with (and without) EV for 6 h before either being lysed for western blotting, or used in leucocyte attachment assays.
Results: EV from "TNFα" and "TNFα & hypoxia" treated 3T3-L1 cells, increased vascular cell adhesion molecule (VCAM) protein expression in HUVEC, an effect that could be blocked in a dose-dependent fashion using an anti-TNFα neutralising antibody. No changes in the expression of other HUVEC adhesion molecules (E-selectin, P-selectin, platelet endothelial cell adhesion molecule (PECAM) and VE-Cadherin) were observed in any treatment group. Pre-incubation of HUVEC with "TNFα" EV and "TNFα & hypoxia" EV increased the subsequent attachment of freshly isolated leukocytes.
Conclusion: EV derived from adipocytes cultured in conditions mimicking that of obese adipose tissue, induce VCAM expression in vascular endothelial cells in a TNFα-dependant manner. This predisposes endothelial cells to the subsequent attachment of leukocytes. Preventing the adipocyte EV-induced upregulation of endothelial VCAM may offer a novel therapeutic window for minimising vascular disease in obese patients.

PS05.04

Diabetes affects extracellular vesicle content and function

Makon-Sébastien Njock[1], Mark Chandy[2], Shawn C. Veitch[2], Dakota Gustafson[2], Zhiqi Chen[2], Kim Connelly[3], Mansoor Husain[2] and Jason E. Fish[2]

[1]Laboratory of Molecular Angiogenesis, GIGA Centre, University of Liège, Belgium; [2]Toronto General Hospital Research Institute, University Health Network, Toronto, Canada; [3]Keenan Research Centre for Biomedical Science, St. Michael's Hospital

Introduction: The role of circulating extracellular vesicles in cardiovascular diseases remains incompletely understood. We previously demonstrated that extracellular vesicles circulating in plasma of healthy mice can suppress monocyte activation through transfer of anti-inflammatory microRNAs. Here we set out to determine the effect of diabetes on the function of plasma extracellular vesicles since diabetes is known to negatively affect vascular function, playing a contributory role in cardiovascular diseases.
Methods: Circulating plasma extracellular vesicles were isolated from mouse and rat models of type 2 diabetes. Extracellular vesicles were characterised with nanoparticle tracking analysis. Furthermore, qPCR and RNA-sequencing approaches were used to characterise vesicle content and function.

Results: We found that vesicle abundance and size were increased in mouse and rat models of type 2 diabetes. MicroRNAs in plasma extracellular vesicles were dysregulated during the progression of diabetes in these models. Finally, we demonstrate that vesicles isolated from diabetic plasma can activate inflammatory pathways in endothelial cells. Current studies are seeking to determine the contribution of microRNA transfer to endothelial dysfunction.
Conclusions: These studies suggest that the microRNA content and function of extracellular vesicles are dysregulated during diabetes. Advancements in this area could facilitate the development of more effective non-invasive diagnostics, prognostics, and therapeutics.
Funding: Supported by funding from the Canadian Vascular Network and the Canadian Institutes of Health Research.

PS05.05

Intra-cardiac release of extracellular vesicles governs infiltrating monocyte activation following myocardial infarction

Xavier Loyer[1], Ivana Zlatanova[1], Min Yin[1], Kiave-Yune HoWangYin[1], Cecile Devue[1], Phatchanat Klaihmon[1], Coralie L Guerin[2], Marouane Kheloufi[1], Jose Vilar[1], Bernd Fleischmann[3], Philippe Menasché[4], Jean-Sebastien Silvestre[1] and Chantal M Boulanger[1]

[1]Inserm UMR970 – Paris Cardiovascular Research Centre (PARCC); [2]National Cytometry Platform, Department of Infection and Immunity, Luxembourg Institute of Health; [3]Institute of Physiology, University of Bonn, Life and Brain Centre, Medical Faculty, Germany; [4]Inserm UMR970 – Paris Cardiovascular Research Centre (PARCC), Department of Cardiovascular Surgery, Hôpital Européen Georges Pompidou, APHP, Paris, France

Introduction: A rapid and massive influx of inflammatory cells occurs into ischemic areas following myocardial infarction (MI). This results in the local release of cytokines and growth factors, but the mechanisms regulating their production are not fully explored in the ischemic myocardium. Extracellular vesicle (EV) release in the interstitial space curbs important biological functions, including inflammation. So far, there is no evidence of EVs in situ release in the heart following MI. The present study tested the hypothesis that local generation of EVs in the infarcted heart coordinates cardiac inflammation following MI.
Methods: MI was induced by permanent left anterior descending artery ligation in C57BL/6 mice. Sham-operated mice were used as controls. Sham and MI mice were sacrificed between 0 and 3 days after the onset of ischemia. EVs from ischemic and sham left ventricles were isolated by sequential centrifugations, and separated into microvesicle-enriched (MVs) and exosome-enriched (Exos) fractions. Both fractions were analysed by TRPS (qNANO). In addition, MVs cellular origin and phosphatidylserine exposure were determined by flow cytometry. FACS-sorted Ly6 C+ monocytes were isolated from ischemic myocardium 24 h post-ligation and were exposed *in vitro* for 24 hours to either MVs or Exos isolated from MI hearts 24 h post ligation. ELISA assessed subsequent cytokine release from cardiac-derived monocytes.
Results: Coronary artery ligation in mice transiently increases EV levels in the left ventricle when compared to sham animals. EVs from infarcted hearts were characterised as MVs and Exos based on their size (Exos mean diameter 118 ± 4 nm vs MVs 252 ± 18 nm). Exos fraction was enriched in the exosomal markers CD63 and CD9. MVs were transiently release at 15 and 24 h post ischemia and then returned to shams levels. Exosomes follow similar time-dependent pattern. MVs mostly originated from cardiomyocytes and increased the release of IL-6, chemokines CCL2, CCL7 and interleukin-10 from FACS-sorted Ly6C+ cardiac monocytes. Exos stimulated the release of anti-inflammatory IL-10 only.
Conclusion: These results highlight the paracrine crosstalk between cardiac inflammatory cells and endogenously released extracellular vesicles following myocardial infarction.

PS05.06

Acoustic trapping of microparticles and its application in measuring the effect of bilberry powder consumption on plasma microparticles in patients with myocardial infarction

Paulina Bryl-Gorecka[1], Ramasri Sathanoori[1], Rikard Landberg[2], Mikael Evander[3], Björn Olde[1], Thomas Laurell[4], Ole Fröbert[5], David Erlinge[1] and Lilith Arevström[5]

[1]Department of Cardiology, Clinical Sciences, Lund University, Sweden; [2]Swedish University of Agricultural Sciences, Uppsala, Sweden; [3]Department of Biomedical Engineering, Lund University, Sweden; [4]Lund University; [5]Faculty of Health, Department of Cardiology, Örebro University, Sweden

Introduction: Microparticles (MPs) are submicron particles released under several physiological and pathological states. Isolation, enumeration and characterisation of MPs from human plasma are promising approaches to understand their role in pathophysiology of cardiovascular diseases (CVD). Current methods are time consuming, and require large sample volumes, which is a limitation when analysing clinical samples. Here we have developed an alternative method based on a microscale acoustic standing wave technology to retain particles in a microfluidic channel. Bilberries (*Vaccinium myrtillum*) are known to improve endothelial function and the antioxidant status. In order to understand the effect of bilberry powder consumption on circulating MPs, we analysed plasma samples from myocardial infarction (MI) patients before and 8 weeks after bilberry powder consumption.
Methods: All participants provided informed consent and the study was approved by the regional ethics review committee in Uppsala, Sweden. MPs were isolated from diluted cell free plasma using acoustic trapping. Briefly, exciting a capillary with a piezoelectric transducer creates a standing wave that traps MPs together with 12 mm polystyrene seed particles through acoustic radiation forces. We used an internal control of pooled frozen plasma from healthy volunteers to validate device performance. MPs originating from endothelial cells (EMPs) and platelets (PMPs) were enumerated using flow cytometry.
Results: The acoustic trapping method achieved ±52% recovery of CD42a+ MPs using the internal control. Isolation and enumeration of EMPs and PMPs in the patient samples showed that bilberry powder consumption for 8 weeks decreased the levels of both types of MPs.
Summary: The acoustic trapping is a fast and efficient method for plasma MP isolation. It uses small sample volumes, making it a promising solution in clinical practice. Bilberry extract consumption decreases EMP and PMP levels in patients with myocardial infarction.
Financial support: This work was supported by Swedish Foundation for Strategic Research, Knut and Alice Wallenberg Foundation and Örebro University Hospital Research Foundation.

PS05.07

The signature of apoptotic endothelial cell-derived microparticles in patients with different phenotypes of chronic heart failure

Alexander E. Berezin[1], Alexander Kremzer[2], Tatyana Samura[2] and Tatyana Berezina[3]

[1]Internal Medicine Department, Medical University, Zaporozhye, Ukraine; [2]Clinical Pharmacology Department, Medical University, Zaporozhye, Ukraine; [3]Private Hospital VitaCenter, Zaporozhye, Ukraine

Introduction: Chronic heart failure (HF) remains a leading cause of cardiovascular (CV) mortality and morbidity worldwide. The aim of the study was to investigate whether the pattern of endothelial progenitor cells (EPCs) with angiogenic capacity and apoptotic endothelial cell-derived microparticles (EMPs) would be able to differentiate HF with reduced (HFrEF) and preserved (HFpEF) ejection fraction.
Methods: One hundred and sixty-four chronic HF subjects met inclusion criteria. Patients with global left ventricular ejection fraction 50–59% were categorised as the HFpEF group (n = 79) and those with ≤45% as the HFrEF group (n = 85). The flow cytometric technique was used for predictably distinguishing circulating cell subsets depending on expression of CD45, CD34, CD14, Tie-2 and CD309 antigens and determining endothelial cell-derived microparticles. CD31+/annexin V + was defined as apoptotic endothelial cell-derived MPs, MPs labelled for CD105+ or CD62E+ were determined as MPs produced due to activation of endothelial cells.
Results: In multivariate logistic regression model T2DM (R^2 = 0.26, p = 0.001), obesity (R^2 = 0.22, p = 0.001), previous MI (R^2 = 0.17, p = 0.012), galectin-3 (R^2 = 0.67, p = 0.012), CD31+/annexin V+ EMPs to $CD14^+CD309^+$ cells ratio (R^2 = 0.16, p = 0.001), $CD31^+$/annexin V^+ EMPs (R^2 = 0.11, p = 0.001), NT-proBNP (R^2 = 0.11, p = 0.046), CD31+/annexin V+ EMPs to $CD14^+CD309^+Tie\text{-}2^+$ cells

ratio (R^2 = 0.102, p = 0.001), CD14+CD309+ cells (R^2 = 0.058, p = 0.001) and $CD14^+CD309^+$ Tie-2^+ cells (R^2 = 0.044, p = 0.028) were found as independent predictors of HFpEF. Using multivariate Cox-regression analysis adjusted aetiology (previous myocardial infarction), cardiovascular risk factors (obesity, type 2 diabetes mellitus) we found that NT-proBNP (OR 1.08, 95% CI = 1.03–1.12 p = 0.001) and CD31+/annexin V + EMPs to $CD14^+CD309^+$ cells ratio (OR 1.06, 95% CI = 1.02–1.11, p = 0.02) remained independent predictors for HFpEF.
Conclusion: We found that CD31+/annexin V+ EMPs to CD14+CD309 + cells ratio added to NT-proBNP, clinical data, and cardiovascular risk factors has exhibited the best discriminate value and higher reliability to predict HFpEF compared with NT-proBNP and clinical data /cardiovascular risk factors alone.

PS05.08

Identification and characterisation of exosomes derived from blood outgrowth endothelial cells in oxidative stress conditions

Arief Wibowo and Stefan Janssens

KU Leuven

Introduction: Blood outgrowth endothelial cells (BOECs) mediate therapeutic neovascularisation in experimental models. We hypothesised that BOECs promote angiogenesis and protect against oxidative stress via secretion of exosomes.
Methods: BOECs were isolated from the peripheral blood of patients with severe ischemic heart disease and were exposed to hypoxia (1% O_2) or normoxia (21% O_2) for 12 h. Exosomes were isolated from the medium by differential ultracentrifugation. Size and the number of exosomes were determined by nano tracking analysis (NTA) and immunoblot analysis of the surface markers, TSG101 and Flotilin-1. Matrigel 2D tube formation assay was performed to explore angiogenic potential of HUVECs in the presence or absence of BOECs-derived exosomes. qPCR analysis was performed to investigate transcript level of angiogenic factors both in BOECs and exosomes. In addition, H9C2 rat cardiomyoblast cells were incubated with PKH67 labelled BOEC-derived exosomes for 4, 8 and 12 h to assess exosomes uptake. Exosomes mediated protection against H_2O2-induced oxidative stress in H9C2 cells was determined by ROS formation and LDH release.
Results: Quantification of exosomes by NTA showed more exosomes in the medium after hypoxia compared to normoxia ($14.01 \pm 0.23 \times 10^8$ vs. $12.51 \pm 0.23 \times 10^8$ particles/ml). Western blot showed exosome markers TSG101 and Flotilin-1. 2D-Tube formation assay indicated an increased mature vascular network after 4 h exposure to BOECs-derived exosomes from both normoxic and hypoxic conditions compared to negative control ($p < 0.001$). qPCR analysis demonstrates an expression levels of 40–60% for angiogenic factors VEGFA, PLGF, MCP-1 and ANG2 in exosomes compared to BOECs. In addition, exosomes uptake studies showed that PKH67-labelled exosomes were taken up by H9C2 cells as early as 4 h. H_2O_2-induced ROS formation (DCF-DA) and LDH release were greatly attenuated in exosomes pre-treated (4 h) group demonstrating exosomes-mediated protection against oxidative stress.
Conclusion: Our results suggest that BOECs-derived exosomes are taken up by neighbouring cells, induce vascular network formation, and protect against oxidative stress. Further research is needed to investigate the underlying mechanism and potential therapeutic applications of exosomes.

PS05.09

Paracrine effect of GATA-4-modified mesenchymal stem cells on the angiogenesis is mediated by the transfer of miRs via exosomes

Min Gong[1], Bin Yu[1], Yigang Wang[1], Muhammad Ashraf[2] and Meifeng Xu[1]

[1]University of Cincinnati, OH, USA; [2]University of Illnois in Chicago, OH, USA

Introduction: We previously reported that GATA-4-overexpressing mesenchymal stem cells ($MSC^{GATA\text{-}4}$) increased angiogenesis in ischemic myocardium via paracrine effect. Here, we investigated whether the paracrine effect of $MSC^{GATA\text{-}4}$ is mediated by the transfer of miRs via exosomes (EXO).
Methods: MSCs were transduced with GATA-4 using the murine stem cell virus retroviral expression and the conditioned medium was collected from $MSC^{GATA\text{-}4}$ ($CdM^{GATA\text{-}4}$) and its control counterpart MSC^{null} (CdM^{null}).
Results:

(1) The total length of capillary-like tubes in human umbilical vein endothelial cells (HUVEC) and the cumulative sprout length per HUVEC spheroid treated with $CdM^{GATA\text{-}4}$ were significantly longer than in those treated with CdM^{null}. However, the tube-like formation was reduced significantly in the CdM obtained from $MSC^{GATA\text{-}4}$ which treated with GW4869 (10μM), an EXO release inhibitor.
(2) The tube formation was significantly increased in HUVECs treated with EXO isolated from $CdM^{GATA\text{-}4}$ ($Exo^{GATA\text{-}4}$) than in those treated with EXO derived from CdM^{null} (Exo^{null}). The cumulative sprout length per spheroid was also greater in $Exo^{GATA\text{-}4}$-treated HUVECs than Exo^{null}-treated cells. Subcutaneous transplantation of Matrigel plug into mice showed that blood flow and the expression of endothelial cell marker CD31 was significantly increased in the plugs containing $Exo^{GATA\text{-}4}$ (100 μg/plug) than in plugs containing Exo^{null} or BSA control.
(3) Real-time PCR indicated that let-7 family is significantly upregulated in $Exo^{GATA\text{-}4}$ compared to that in Exo^{null}. EXO pre-labelled with PKH26 to track their fate indicated that EXO could be internalised by HUVECs. The expression of let-7 was significantly upregulated in HUVECs treated with $Exo^{GATA\text{-}4}$.
(4) Gain-and-loss function studies indicated that the tube-like structure formation following different EXO treatment was positively related to the expression of let-7f in HUVECs. Moreover, thrombospondin 1 (THBS1), an anti-angiogenic gene, was down-regulated in HUVEC treated with $Exo^{GATA\text{-}4}$ compared with that treated with Exo^{null}.

Conclusion: These results suggested that the increased pro-angiogenic capacity of $MSC^{GATA\text{-}4}$ is associated with angiogenetic miRs transfer via exosomes, which results in regulating the expression of angiogenetic biomolecules in endothelial cells.

PS05.10

Extracellular vesicles-associated and plasma fatty acid binding protein 4 (FABP4) fluctuations following bariatric surgery

Justyna K. Witczak[1], Thinzar Min[2], Sarah Prior[2], Jeffrey Stephens[2], Philip James[3] and Aled Rees[1]

[1]Cardiff University, Cardiff, United Kingdom; [2]Diabetes Research Group, Swansea University, Swansea, United Kingdom; [3]Cardiff Metropolitan University, Cardiff, United Kingdom

Introduction: Bariatric surgery markedly reduces fat mass which results in various cardiometabolic benefits but the effect of weight loss on circulating extracellular vesicles (EVs) remains unclear. We sought to characterise changes in circulating EVs in patients undergoing bariatric surgery with particular focus on changes in adipokine content of plasma EVs.
Methods: Plasma EVs were isolated by differential ultracentrifugation from individuals undergoing bariatric surgery (n = 20, BMI = 54.1 ± 12.6 kg/m^2) at baseline, 1- and 6-months postoperatively. EV concentration was established using Nanoparticle Tracking Analysis. EV origin (CD9: exosome, CD41: platelet, CD235a: erythrocyte, CD11b: leucocyte, CD144: endothelial), cytokine (interferon γ, interleukin-6, TNFα) and adipocyte marker (adiponectin, FABP4, PPARγ) expression was measured by 96-well plate immunophenotyping assay, and compared with plasma adipocytokine levels.
Results: EV concentration and distribution of the principal EV cell-of-origin markers (CD41, CD235a, CD11b, CD144) did not alter in response to surgery, however a significant reduction in EVs measuring between 100 and 200 nm in diameter at 6 months compared to baseline was observed ($p < 0.001$). EV-derived Fatty Acid Binding Protein 4 (FABP4) increased at 1 month (by 49%) before returning to baseline by 6 months (−51.2%, $p < 0.05$), corresponding to similar changes in circulating plasma FABP4 (+21.9% and −24.1% at 1 and 6 months, respectively, $p < 0.001$). Plasma FABP4 also correlated with plasma free fatty acids (FFA's) at 1 month ($p < 0.05$). There were no differences in EV-expressed interferon ϒ, interleukin-6, TNFα, adiponectin, PPARϒ

or CD9. Plasma concentration of IL-6 and adiponectin did not differ either (p = ns). CD9 expression correlated with EV-expressed FABP4 (r = 0.5, $p < 0.001$), adiponectin (r = 0.59, $p < 0.0001$), TNFα (r = 0.53, $p < 0.0001$) and interferon ϒ (r = 0.41, $p < 0.005$) suggesting an EV population of exosomal rather than microvesicle origin transports these proteins predominantly.
Conclusion: Transient rise in EV-associated and plasma FABP4 secretion following bariatric surgery reflects postoperative changes in adipose tissue homeostasis which are most likely triggered by increased lipolysis.

PS05.11

Small EVs related to T-cell-mediated inflammation and vascular function are increased in familial hypercholesterolemia

Morten Hjuler Nielsen[1], Rikke Baek[2], Malene M. Jorgensen[2] and Aase Handberg[1]

[1]Department of Clinical Biochemistry, Aalborg University Hospital, Aalborg, Denmark; [2]Department of Clinical Immunology, Aalborg University Hospital, Aalborg, Denmark

Introduction: Low-grade inflammation and endothelial dysfunction predisposes to atherosclerosis in familial hypercholesterolemia (FH), especially when associated with high levels of oxLDL cholesterol. Activation of both innate and adaptive immune responses against oxLDL is the major cause of inflammation, and extracellular vesicles (EVs) released from both non-immune and immune cells may have important roles in the pathogenesis. The aim of our study was to investigate small EVs derived from endothelial cells and cells involved in the adaptive immune response in subjects with subclinical atherosclerosis.
Methods: Thirty FH patients and 23 controls were included. Intima-media thickness (IMT), a marker of subclinical atherosclerosis, plasma levels of oxLDL and two inflammatory markers (CRP and IL-6) were determined. The EV Array was used to phenotype small EVs. The array containing antibodies targeting proteins expressed on both B- and T-cells, as well as endothelial cells, captured small EVs/exosomes, which were visualised by a cocktail of biotin-conjugated CD9, CD63 and CD81 antibodies. The study was approved by the local ethical committee and informed consent was obtained before inclusion.
Results: FH patients had significantly higher IMT, and higher levels of oxLDL and IL-6. In addition, FH patients had significantly higher level of EVs expressing exosome specific markers CD9, CD63 and CD81, but not TSG101, Alix or Flotilin-1. The T-cell-specific markers CD28, CD4 and CTLA-4 were increased in FH, whereas B-cell-specific markers CD19 and CD80 were unaltered. The endothelial cell-specific markers E-selectin, VE-Cadherin, tPA and THBS1 were increased in FH, whereas VCAM-1 and MCAM were unaltered.
Conclusion: Our findings support increased activity of cells involved in adaptive immunity and endothelial dysfunction in subclinical atherosclerosis. Further studies may improve our understanding of pathophysiology and holds the potential to provide better risk assessment in the future.

PS05.12

Exosomal miRNA profiles in patients with coronary artery disease

Melanie Maerte[1], Dominik Buschmann[2], Marlene Reithmair[3], Florian Brandes[1], Benedikt Kirchner[2], Alexander Chouker[1], Michael Pfaffl[2] and Gustav Schelling[1]

[1]Department of Anaesthesiology, University Hospital, Ludwig-Maximilians-University, Munich, Germany; [2]Division of Animal Physiology and Immunology, TUM School of Life Sciences Weihenstephan, Technical University Munich, Germany; [3]Institute of Human Genetics, University Hospital of Ludwig-Maximilians-University Munich, Munich, Germany

Introduction: Cardiovascular diseases (CVDs) are the number 1 cause of death globally. People with CVDs or who are at high cardiovascular risk need early detection and concise management. Liquid biopsies assessing exosomal microRNA (exmiRNA) profiles could represent a useful tool for diagnosis and monitoring of patients with CVDs. We aimed at identifying differentially regulated exmiRNAs between patients with coronary artery disease (CAD, n = 6) and age and gender matched healthy controls (HCs, n = 7), and detecting previously unknown CAD-associated exmiRNAs.
Methods: Exosomes were isolated from serum samples. The presence of exosomes was confirmed by electron microscopy, nanotracking analysis and western blot. ExmiRNAs were profiled by next-generation sequencing. Informed consent was obtained from all study subjects and the investigation was approved by the local IRB.
Results: In CAD patients, a total of 5 and 11, respectively, distinct exmiRNAs were down- and upregulated compared to HCs. With the exception of miR-320a, a known key regulator of multiple aspects of atherogenesis, these differentially regulated exmiRNAs had not been previously associated with CAD. *In silico* analysis demonstrated target genes of the identified exmiRNAs with critical regulatory functions in CVDs. These included serum response factor (SRF), hypoxia-dependent regulators of myocardial cell differentiation (MYOCARDIN), risk factors for atherosclerotic vascular disease (TGBR2) and IGF1R signalling, an important predictor of myocardial stem cell growth potency and outcome of cardiac revascularisation surgery.
Conclusion: This study identified miRNA expression profiles from blood-derived exosomes in CAD suggesting novel approaches to diagnosis and monitoring of patients with CVDs.

PS05.13

Preconditioning affects the miRNA composition of cardiac cell-derived EVs

Sebastian Borosch[1], Eva Dahmen[1], Christian Stoppe[2], Eva Buhl[3], Christian Beckers[1], Bernd Denecke[4], Andreas Goetzenich[1], Rüdiger Autschbach[1] and Sandra Kraemer[1]

[1]Department of Thoracic and Cardiovascular Surgery, University Hospital RWTH Aachen, Aachen, Germany; [2]Department of Intensive Care Medicine, University Hospital RWTH Aachen, Aachen, Germany; [3]Electron Microscopy Facility, University Hospital RWTH Aachen, Aachen, Germany; [4]Interdisciplinary Centre for Clinical Research, University Hospital RWTH Aachen, Aachen, Germany

Introduction: To protect the heart from ischemia reperfusion injury, preconditioning with brief cycles of hypoxia or volatile anaesthetics like isoflurane is a promising technique. In this context, communication between different cardiac cells and their exchange of proteins, lipids and especially miRNA by EVs has come into focus of our research. miRNAs have been shown to facilitate cardioprotective properties by inducing cardiomyocyte proliferation or regulating neovascularisation. However, it still remains elusive whether specific preconditioning stimuli trigger the release of EVs with cardioprotective properties.
Methods: Primary rat cardiac fibroblasts were preconditioned with either isoflurane (1.5 Vol%, 4 h) or hypoxia (<1% O_2, 1 h). Supernatants were collected after 48 h and EVs were isolated by size-exclusion chromatography. Particles were characterised by tunable resistive pulse sensing (TRPS) and electron microscopy. miRNA was isolated from EVs and an affymetrix miRNA microchip array was performed.
Results: Preconditioning triggered the release of EVs with an altered miRNA composition compared to untreated cardiac fibroblasts even though vesicle number was not affected. Microarray analysis revealed that preconditioning with isoflurane significantly regulated 14 miRNAs. Out of these, 9 (e.g. miR-351 (p = 0.008), miR-384-3p (p = 0.04) and miR-352 (p = 0.01)) have been described in the context of cardioprotection. Hypoxic stimulation affected 11 miRNAs in total and out of these 7, for example miR-761 (p = 0.04), with cardioprotective properties. Additionally, several miRNAs from the let 7 family like let-7a-1-3p (p = 0.01) were significantly regulated by both treatments.
Conclusion: The miRNA cargo released after a preconditioning stimulus is strongly dependent on the applied stimulus indicating different sorting and loading mechanisms. Preconditioning probably influences the loading of cardioprotective miRNAs in EVs which in turn might indicate a role in cardioprotection.

PS05.14

Characterisation of inside-out erythrocyte microvesicles in sickle cell blood

Rachel A. Smith[1], Tosti Mankelow[2], Rebecca Griffiths[2], Sara Trompeter[3] and David Anstee[2]

[1]University of Bristol, United Kingdom; [2]NHSBT; [3]University College Hospitals London, United Kingdom

Introduction: Elevated levels of circulating red cell microvesicles (RMVs) have been observed in sickle cell disease (SCD) patients. These RMVs express phosphatidylserine (PS) which is thought to contribute to the pro-inflammatory state associated with SCD. The majority of studies on SCD RMVs have only measured Glycophorin A (GPA) expression but did not examine other membrane proteins. Recently, "inside-out" microvesicles were observed to be present in SCD erythrocytes (1). To examine whether "inside-out" microvesicles can be detected in SCD plasma, this study examines the expression of intracellular (IC) domains of red cell membrane proteins on the surface of RMVs.
Methods: Blood was collected from SCD patients receiving regular transfusion therapy. RMVs attached to red cells were analysed by confocal microscopy and RMVs in plasma were analysed by flow cytometry using Annexin V and fluorescent labelled antibodies against IC domains of GPA and C, Anion exchanger-1 (Band 3), and Glucose transporter-1 (GLUT-1). Size distribution of RMVs was assessed by flow cytometry using commercial standards.
Results: In agreement with published results (1), SCD patients had elevated numbers of red cells with an attached RMV which stained positive for IC protein domains, compared to healthy donors. This indicates that these RMVs have an inside-out orientation. RMVs in SCD plasma were found to exist in two distinct populations. Both populations expressed PS alongside extracellular GPA and Band 3 and were shown to be >0.5 μm to <1 μm in size. However, one population also stained positively for IC domains of GPA and C, Band 3, and GLUT-1. This sub-population is present in negligible amounts in plasma from healthy donors.
Conclusion: This study is the first to examine the presence of IC membrane proteins on RMVs in plasma from SCD patients. A subset of plasma RMVs were found to stain positively for IC domains of red cell proteins. However, these RMVs also expressed extracellular protein domains so it is unclear whether the RMV membranes are inside-out or these microvesicles, once released from reticulocytes, become permeable to antibodies. The RMVs in plasma are smaller than inside-out vesicles emerging from reticulocytes suggesting membrane instability in the circulation.

Reference

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LBP.46

miR-193 is released by cardiomyocytes in response to stress and inhibit fibroblast proliferation and activation

Mun Chun Chan[1], Olivia Ziegler[2], Rodosthenis Rodosthenous[3], Kirsty Danielson[4], Ravi Shah[3] and Saumya Das[2]

[1]Georgetown University, DC, USA; [2]Mass General Hospital, MA, USA; [3]MGH; [4]University of Dunedin, New Zealand

Introduction: Plasma microRNA-193 appears to be increased in human patients with cardiomyopathies and after cardiac injury. However, its functional role in modulating cardiac remodeling has not been studied. Previous studies have shown intercellular communication between cardiac myocytes and fibroblasts. Here we describe the regulation of miR-193a primary cardiomyocytes and define its role in mediating signaling between cardiac myocytes and fibroblasts.
Methods: Experiments were conducted with isolated neonatal rat ventricular myocytes (NRVMs) and neonatal rat cardiac fibroblasts (CFs). The regulation of miR-193 in NRVMs in response to hypoxia/re-oxygenation and its effects on CF phenotype was studied. LNA-miR193a mimic (gain-of-function) and LNA-anti-miR193a (loss-of-function) were used to further analyze the role of miR-193a in mediating intercellular signaling between NRVMs and CFs.
Results: 1. NRVM expression and secretion of miR-193a in EVs increases in response to hypoxia/re-oxygenation. **2**. CFs treated with hypoxia-treated NRVM conditioned-media show an increase in miR-193a levels (but not pre-miR-193a), and this is abrogated by pre-treatment of NRVM-derived EVs with RNAse in combination with detergent. **3**. Treatment of CFs with CM-conditioned medium and EVs leads to inhibition of CF proliferation and activation in response to TGF-beta and angiotensin II. This phenotype is recapitulated with direct transfection of miR-193a mimic, but abrogated by silencing of miR-193a in NRVMs. **4**. Bioinformatics analysis identified several targets of miR-193a and qRT-PCR confirmed a marked decrease in ERBB4 and ARHGAP19 at the mRNA and protein level suggesting novel regulatory pathways in fibroblast biology in the context of cardiac remodeling.
Summary/Conclusion: Extracellular miR-193a is altered in human cardiovascular diseases and appears to be dynamically regulated by the stress of hypoxia/re-oxygenation in cardiac myocytes. miR-193a appears to be secreted in EVs and may mediate intercellular communication between myocytes and fibroblasts to regulate fibrosis, a critical process in cardiac remodeling.
Funding: NIH NCATS UH3TR000901 to SD.

Poster Session S06 – EVs in Cancer Biology and Progression
Chairs: Lucia Languino and TBD 5:15–6:30 p.m.

PS06.01

Significant increase of blood exosomes in pulmonary vein as potential prognostic biomarker for lung cancer patients

Byeonghyeon Choi[1], Yu Hua Quan[2], Jiyun Rho[2], Xu Rong[2], Kook Nam Han[2], Sunghoi Hong[3], Yeonho Choi[4], Yong Park[5], Ji Ho Park[6], Young Ho Choi[2] and Hyun Koo Kim[2]

[1]Korea University; [2]Department of Thoracic and Cardiovascular Surgery, Korea University Guro Hospital, Korea University College of Medicine, Seoul, Republic of Korea; [3]School of Biosystem and Biomedical Science, Korea University, Republic of Korea; [4]Department of Bioconvergence Engineering, Korea University, Republic of Korea; [5]Division of Haematology-Oncology, Department of Internal medicine, Korea University Anam Hospital, Korea University College of Medicine, Republic of Korea; [6]KAIST, Republic of Korea

Introduction: Previous researches have demonstrated that the level of exosomes is increased in cancer patients than healthy controls. This study was conducted to evaluate the variation of exosome-count in the proximal tumour-drainage vessel and peripheral vessels during surgery for VX2 rabbit lung cancer model and primary lung cancer patients.
Methods: A total of 6 rabbits were used in this study (3 in normal group, 3 in lung cancer group). Rabbit VX2 lung cancer model was made by computed tomographic (CT) guided injection of VX2 cancer. Blood was sampled from rabbit ear vein (peripheral vein) and pulmonary vein (proximal tumour-drainage vein). A total of 3 controls and 6 patients with primary lung cancer who had pT2aN0 and underwent lobectomy were selected. For each patient, 3 ml of blood was sampled from the radial artery before surgery and from pulmonary vein during surgery. Normal blood was collected from peripheral vessels. Exosomes were isolated by serial centrifugation followed by ExoQuick and quantitative analysis was performed by NTA and western blot.
Results: Exosome-count was not different in normal rabbits according to blood sampling sites (peripheral: 2.78×10^8 particles/ml, pulmonary: $2.64 \times v10^8$ particles/ml, $p = 0.104$). But, in VX2 rabbit lung cancer model, exosomes were increased by 623.5% in peripheral vein (1.73×10^9 particles/ml, $p = 0.003$) and 787.9% in pulmonary vein (2.08×10^9 particles/ml, $p = 0.001$) comparing to those of normal. And, we confirmed that exosomes in VX2 lung cancer model was increased by 120.0% ($p = 0.05$) on the proximal tumour-drainage vein than peripheral vessel. In human blood, peripheral blood exosomes were increased by 216.7% in cancer patient in comparison with controls (2.44×10^8 particles/ml in control, 5.3×10^8 particles/ml in patients, $p = 0.04$). And, exosomes were significantly increased by 542.1% in pulmonary vein (3.0×10^9 particles/ml, $p = 0.01$) comparing to peripheral vessels in cancer patients.
Summary: We firstly demonstrated that the increase of exosome was more prominent in tumour-drainage veins than peripheral vein in animal cancer models and lung cancer patients. We suggest that increase of exosomes from tumour-drainage veins may provide more relevant prognostic information of the lung cancer patients comparing to those from peripheral vein after surgery.

PS06.02

The effect of erythrocyte-derived microvesicles on the malignant potential of gastric and colorectal cancer

Daiki Matsubara, Tomohiro Arita, Daisuke Ichikawa, Hirotaka Konishi, Katsutoshi Shoda, Shuhei Komatsu, Atsushi Shiozaki, Shinpei Ogino, Yuji Fujita, Toshiyuki Kosuga, Hitoshi Fujiwara, Kazuma Okamoto and Eigo Otsuji

Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan

Introduction: Exosomes are small membrane vesicles of endocytic origin secreted by most cell types. They play important roles in intercellular communications and also influence cancer survival or outgrowth in malignancies. Besides, several studies suggest that bone-mallow-derived cell, such as many types of blood cells, contribute to cancer development. In this study, the effects of erythrocyte-derived microvesicles (EDMs) on the progression of gastric and colorectal cancer cells were investigated.
Methods: Peripheral blood samples of gastric and colorectal cancer patients were collected before surgical resection. After centrifugation of whole blood, erythrocytes were cultured in exosome-free medium for 24 h. The culture medium was filtered through 0.22 μm filter and ultracentrifuged. An obtained pellet was washed with PBS, and ultracentrifuged again. The Pellet was resuspended in PBS and stored at −80° C. Presence and deliveries into various cells of EDMs were confirmed by immunofluorescence staining. The effects of EDMs on the malignant potential of gastric and colorectal cancer cells were investigated in invasion, migration and wound healing assays.
Results: EDMs were labelled with PKH67 and the delivered EDMs were detected in the cellular cytoplasm using fluorescence microscopy. EDMs obtained from colon cancer patients enhanced the motility of colon cancer cells in wound healing assays. Invasion and migration assays were also performed for gastric cancer cells with gastric cancer patients-derived EDMs. These assays revealed that EDMs obtained from gastric cancer patients enhanced the migration ability of gastric cancer cells.
Conclusion: Microvesicles secreted from erythrocytes of gastric and colorectal cancer patients may promote the malignant potential. Further studies are in progress to fully elucidate the role of EDMs in cancer survival or outgrowth.

PS06.03

Isolation and characterisation of extracellular vesicles from patient-derived primary high-grade serous ovarian cancer cells

Laura Lehtinen[1], Parvez Syed[2], Rainer Lehtonen[3], Sampsa Hautaniemi[3], Aled Clayton[4] and Olli Carpèn[5]

[1]Department of Pathology, University of Turku and Turku University Hospital, Turku, Finland, [2]Department of Biochemistry/Biotechnology, University of Turku, Finland, [3]Research Programmes Unit, Genome-Scale Biology, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [4]Division of Cancer and Genetics, School of Medicine, Cardiff University and Velindre Cancer Centre, Cardiff, United Kingdom, [5]Department of Pathology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

Introduction: Ovarian cancer is prime example of a disease, in which increased molecular knowledge has not yet translated to outcome improvement: therefore novel approaches are needed. Most studies on high-grade serous ovarian cancer (HGS-OvCa) concentrate on the genetic background and characterisation of cell subpopulations inside the tumours, while a critically important step in disease progression, the discussion between tumour cells and surrounding stroma, has gained less attention. According to current knowledge, extracellular vesicles (EV) provide intercellular communication between tumour and stromal cells. The content of EVs shed by cancer cells differ from normal cells, but the correlation with tumour characteristics and clinical data remains unknown.
Methods: Primary ovarian cancer cell lines were established from fresh HGS-OvCa tumours and ascites fluids. The study protocol and use of all material was approved by local ethical committees and comply with the Declaration of Helsinki. For isolation of EVs, primary cells were cultured in Integra bioreactor flasks, conditioned culture media was collected and subjected to sequential centrifugations and filtering followed by ultracentrifugation with sucrose cushion. The isolated EVs were analysed with nanoparticle tracking analysis and transmission electron microscopy, and characterised for the presence of protein markers with western blotting and ELISA assays. For further characterisation, RNA was extracted from the EVs and the cargo composition explored with Next-generation sequencing. RNAseq data was also obtained from the cell lines and original tumours.

Bioinformatic analyses are currently performed to compare the EV RNA profile to the original cells and tumour samples.
Results: We have successfully isolated significant amounts of highly pure EV samples from primary ovarian cancer cells. Preliminary results indicate variance in EV shedding between different primary cell lines. In addition, analysis of surface protein markers showed differences in the expression of Epcam and ITGA3, both with previous implications in malignant tumours.
Conclusion: This study indicates differences in EV composition between HGS-OvCa primary cell lines. Comprehensive results of these analyses will be presented in the meeting.

PS06.04

Extracellular vesicles have a functional role in the aggressive behaviour of young women's and postpartum breast cancer

Troy B. Schedin[1], Kimberly R. Jordan[1], Jessica Hall[1], Kirk Hansen[1], Pepper Schedin[2] and Virginia F. Borges[1]

[1]University of Colorado, CO, USA; [2]Oregon Health & Science University, OR, USA

Introduction: Young women's breast cancer (YWBC) affects 27,000 US women under age 45 annually. Half of these cancers occur within 5 years of childbirth, termed postpartum breast cancer (PPBC), which is associated with a 3-fold increased risk of metastasis and death. Extracellular vesicles (EVs) released by cancer cells are found in the peripheral blood of cancer patients and alter both the local tumour microenvironment and establish distant metastatic niches. EVs isolated from aggressive breast cancer cell lines increase proliferation and invasion of less invasive breast cancer cells *in vitro*. However, the impact of EVs isolated from breast cancer patients is largely unknown. We hypothesised that EVs from YWBC/PPBC patients contain pro-metastatic cargo, influence breast cancer cell behaviour and induce genetic changes in recipient cancer cells.
Methods: EVs were isolated using size-exclusion chromatography (SEC) from plasma of 10 healthy young women and 20 YWBC patients balanced for parity, age, subtype and stage. EV proteins from various clinical groups were compared using a basic proteomics approach and the functional impact of these EVs was determined using tumour cell motility, proliferation, and gene expression assays.
Results: We identified 22 proteins that were significantly increased in the EVs of YWBC compared to the healthy donor group. Eight proteins were significantly increased in PPBC EVs, providing novel breast cancer biomarkers in a clinically high-risk patient cohort. YWBC EVs are engulfed by BC cells *in vitro* and increased the proliferation and invasiveness of ductal carcinoma in situ, DCIS, cells in both 2D scratch wound and 3D organoid assays. Furthermore, gene expression was altered in DCIS cells after exposure to YWBC EVs, demonstrating the functional capability of EVs produced by breast cancer cells.
Conclusion: EVs isolated from YWBC & PPBC cases have unique protein content and increase breast cancer invasiveness. EV's isolated from YWBC & PPBC cases alter gene expression in non-invasive DCIS cells, thereby promoting tumour cell proliferation and invasion. These results suggest potential mechanistic roles for EVs in the increased metastatic risk groups of YWBC and PPBC and provide potential novel candidate targets for intervention.

PS06.05

Analysis of biodistribution and cellular uptake of B16BL6-derived exosomes in relation to their biological effects on tumour progression

Akihiro Matsumoto[1], Yuki Takahashi[1], Makiya Nishikawa[1], Kohei Sano[1], Masaki Morishita[1], Chonlada Charoenviriyakul[2], Hideo Saji[1] and Yoshinobu Takakura[1]

[1]Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan; [2]Kyoto University, Kyoto, Japan

Introduction: A growing body of evidences has revealed that cancer cell-derived exosomes play important pathophysiological roles in tumour progression. However, *in vivo* behaviour of cancer cell-derived exosomes are scarcely investigated so far although it is one of the most important factors in understanding their roles. In the present study, we focused on the biodistribution of exogenously administered exosomes derived from murine melanoma B16BL6 cells in relation to their biological effects on tumour progression.
Methods and Results: Addition of B16BL6-derived exosomes to B16BL6 cells increased proliferation and inhibited apoptosis, which was correlated with the changes in the intracellular amounts of proliferation- and apoptosis-related proteins. Addition of GW4869, an inhibitor of exosome secretion, reduced the proliferation of B16BL6 cells, which was restored by the addition of B16BL6-derived exosomes to cells. After intratumoral injection of radiolabeled B16BL6-derived exosomes to mice, most radioactivity was detected in the tumour tissue. Fractionation of the cells in the tumour tissue revealed that exosomes were mainly taken up by B16BL6 cells. Moreover, intratumoral injection of B16BL6-derived exosomes promoted tumour growth while that of GW4869 suppressed the tumour growth.
Conclusion: These results indicate that cancer cells efficiently take up their own exosomes to induce tumour progression.

PS06.06

Characterisation of DNA from cancer cell-derived extracellular vesicles

Yumi Kawamura[1,2], Yusuke Yamamoto[1], Taka-Aki Sato[2] and Takahiro Ochiya[1]

[1]Division of Molecular and Cellular Medicine, National Cancer Centre Research Institute, Japan; [2]Ph.D. Programme in Human Biology, School of Integrative and Global Majors, University of Tsukuba, Japan

Introduction: The composition of genetic material in extracellular vesicles (EVs) has sparked interest particularly in the potential for horizontal gene transfer by EVs. Several reports have demonstrated the presence of mitochondrial DNA, single-stranded DNA and double-stranded DNA in EVs. However, the localisation of DNA in EVs has been unclear, in addition to their functionality in EV-recipient cells. The aim of this study was to examine the DNA content of cancer cell-derived EVs (termed EV-DNA) in order to understand their physiological significance in the cancer microenvironment.
Methods: EVs were isolated from human cancer cell lines HCT116 and MDA-MB-231 by ultracentrifugation, and characterised by western blot and nanoparticle tracking analysis. EVs were untreated or pretreated with Exonuclease III or DNase I prior to DNA extraction. DNA concentration and size distribution was compared between untreated and pretreated EV groups. EV-DNA was assessed for specific sequences by PCR.
Results: Cancer cell-derived EVs purified by ultracentrifugation were abundant in DNA. For instance, KRAS mutations were present in EV-DNA, reflective of the parental cell lines HCT116 and MDA-MB-231. In addition to this, it was found that high copies of retrotransposon DNA sequences were found in EV-DNA. However, the pretreatment of EVs with Exonuclease III and DNase I before DNA extraction significantly decreased the concentration and size distribution of EV-DNA, indicating that DNA is mostly present on the outer surface of EVs. Interestingly, retrotransposon sequences were detected in EVs after DNase I treatment.
Conclusion: Here we show that DNA is abundant on the outer surface of cancer cell-derived EVs. Although it is still unknown whether EV-DNA can be integrated into the genome of the recipient cell, it is likely that EV-DNA may cause phenotypic changes that promote tumour growth in neighbouring cells. Further investigation into the functionality of genetic material in EVs will help to better define their roles in disease progression and their potential use as circulating biomarkers.

PS06.07

Investigating the involvement of macroautophagy in exosome production

Jing Xu[1], Shane Colborne[1], Elham Hosseini-Beheshti[2], Emma Guns[3], Gregg Morin[1] and Sharon Gorski[1]

[1]Michael Smith Genome Sciences Centre, Canada; [2]Garvan Institute of Medical Research, NSW, Australia; [3]Vancouver Prostate Centre, Vancouver, Canada

Introduction: Macroautophagy (hereafter autophagy) is a cellular catabolic process wherein a portion of cytoplasm containing proteins and organelles is digested via engulfment in double-membrane autophagosomes and subsequent fusion with lysosomes. This degradative role of autophagy can support cell survival under stress by recycling cellular content for energy and building blocks. Autophagy also contributes to many other processes and interacts with other cellular pathways. Autophagosomes are known to fuse with multivesicular bodies (MVBs), sites of exosome biogenesis, to form amphisomes. However, the relationship between autophagy and exosome production remains poorly understood. In triple-negative breast cancers (TNBCs), a subtype of breast cancer lacking oestrogen, progesterone and HER2 receptors, both autophagy flux and exosome production were shown to increase in response to stress. Therefore we investigate the interrelationship between autophagy and exosome release using TNBC as a model.
Methods: TNBC cell line MDA-MB-231 was used for initial characterisation of exosomes and autophagy levels. Conditioned media from 48 h of serum-free incubation was pre-cleared using differential centrifugation and concentrated using centrifugal filtration. ExoQuick reagent was used to precipitate exosomes. Western blotting of known markers, NanoSight and transmission electron microscopy were used to characterise the exosomes isolated. Mass spectrometry was used to identify exosomal proteins.
Results: We observed increased autophagy flux in TNBC cell line MDA-MB-231 after 48 h of serum starvation. Treatment with lysosomal inhibitor chloroquine blocked autophagy and altered exosomal protein cargo. Autophagosome-associated proteins were identified in MDA-MB-231 derived exosomes, suggesting that the autophagy process and exosome release might be interconnected.
Conclusion: Macroautophagy is closely related to the endocytic pathway via the multivesicular body, and may contribute to exosome cargo. Further studies are underway to investigate the exosomal protein content from TNBC cells with both early and late-stage autophagy blockade.

Funding: This work is supported by CIHR in partnership with Avon Foundation for Women.

PS06.08

Packaging of specific mRNA into extracellular vesicles using human endogenous retroviral elements

Kristina P. Friis[1], Romy Verschoor[1], Leonora Balaj[1], Rafael Contreras-Galindo[2], David M. Markovitz[2] and Xandra O. Breakefield[1]

[1]Department of Neurology and Radiology and Program in Neuroscience, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA; [2]Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

Introduction: Human endogenous retroviruses (HERVs) belong to the group of LTR retrotransposons. These highly mobile elements are scattered throughout our genome and are remnants of ancient viral infections. Today HERVs constitute 8% of our genome and help maintain the genetic heterogeneity. Being unable to create replication-competent retroviruses, they are still capable of forming viral-like particles (VLPs).
Interesting, these HERVs have been found to be highly enriched in the tumour extracellular vesicles (EVs) compared to their cells of origin. Previous reports have shown that these mobile elements can be transferred *in vitro* from medulloblastoma cells to normal human umbilical vein endothelial cells (1). Hence, we are now investigating the potential use of these HERV elements to increase the packaging specificity and transfer of mRNA through EVs.
Methods: The assay is based on the packaging and transfer of a neomycin reporter gene (*neo*) using the BOGOTA construct (2). BOGOTA includes parts of the HERV-K packaging signal and has previously been shown to increase the amount of *neo* transferred and hence expressed in VLPs.
Results: Several cell lines have been screened for high HERV expression, and when combined with the expression of BOGOTA preliminary studies indicate that BOGOTA increases the selective packaging of the reporter mRNA into EVs. The selective packaging and transfer is further improved when co-expressing the HERV proteins known to be involved in VLP organisation and packaging, and especially when focusing on the HERV Env-expressing subpopulation of EVs.
Conclusion: The selective packaging of specific mRNA into EVs using BOGOTA in cell lines with a high expression of HERVs supports future studies to determine the role of retrotransposon transfer in tumorigenesis, and as a potential means of generating therapeutic vehicles utilising viral packaging signals.

References

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PS06.09

Extracellular vesicle treatment of cholangiocarcinoma cells affects genes of epithelial-mesenchymal transition and cell survival

Ahmet Canbazoglu[1], Ozlem Kucukoglu[1], Verena Boerger[2], Jan-Peter Sowa[1], Guido Gerken[1], Bernd Giebel[2,3] and Ali Canbay[1,4]

[1]Department of Gastroenterology and Hepatology, University Hospital Essen, University Duisburg-Essen, Essen, Germany; [2]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; [3]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden; [4]Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg, Germany

Introduction: Cholangiocarcinoma (CCC) is a malignancy of epithelial biliary cells with a survival rate of less than 5% at 5-years. The cause for CCC remains mostly unclear.Extracellular vesicles (EVs) produced by healthy or tumour cells are known to affect their cellular environment. In the present study influence of EVs from cholangiocyte cell types on tumorigenesis or metastasis formation via an epithelial-mesenchymal transition (EMT) was analysed.
Methods: EVs released from the H69 cell line, mimicking healthy human cholangiocytes,and from TFK-1 cholangiocarcinoma cells were isolated by ultracentrifugation and characterised by nanoparticle tracking analysis (NTA). H69 and TFK-1 cells each were treated with EVs released from both cell lines separately. Intake of EVs by cells was visualised with PKH67 and PKH26 under a confocal super-resolution microscope (Zeiss, LSM710). Cell proliferation was assessed with MTT assay and gene expression levels of EMT markers (E-cadherin, N-cadherin, S100A4), genes of sonic Hedgehog signalling(PTCH1, Gli), and cell death receptors (DR4, Trail) were analysed by qrtPCR.
Results: EVs secretion of TFK-1 cells was 2.5 fold higher than from H69 cells. Treatment of TFK-1 cells with H69-EVs resulted in a significant reduction of EV release ($p = 0.02$). In parallel TFK-1 cell proliferation was repressed by H69-EVs (MTT). Conversely, TFK-1-EVstreated H69 cells released 1.5 fold more EVs than without treatment. In TFK-1 cells treated with H69-Evs expressions of EMT markers were slightly shifted towards a more epithelial phenotype (n.s.). DR4 ($p = 0.03$) and TRAIL ($p = 0.09$) expressions increased in H69-Evs treated TFK-1 cells. Expression of Gli was reduced ($p = 0.07$). In contrast H69 cells treated withTFK-1 derived EVs exhibited reduced E-cadherin ($p = 0.04$) and elevated N-cadherin ($p = 0.001$) and S100A4 ($p = 0.01$)expressions. Expression of DR4 was decreased in TFK-1-Evs treated H69 cells ($p = 0.03$). The necrosis marker HMGB1 was reduced in H69 cells treated with TFK-1 EVs ($p = 0.002$).
Conclusion: EVs from cholangiocarcinoma cells alter gene expression of cholangiocytes towards apoptosis resistance and an EMT-like gene expression. Conversely, EVs from healthy cholangiocytes increase death receptor expression in cholangiocarcinoma cells.

PS06.10

The role of extracellular vesicles in advanced prostate cancer progression

James D. Riches[1], Irina Oleinikova[2], Lidija Jovanovic[3], The Australian Prostate Cancer Collaboration BioResource[4], Colleen C. Nelson[3], Pamela J. Russell[3] and Carolina Soekmadji[5]

[1]Central Analytical Research Facility, Institute for Future Environments, Queensland University of Technology, Australia; [2]Department of Urology, Queensland Health, Princess Alexandra Hospital, Australia; [3]Australian Prostate Cancer Research Centre-Queensland, Institute of Health and Biomedical Innovation, Queensland University of Technology and Translational Research Institute, Brisbane, Australia; [4]Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Australia; [5]QIMR Berghofer Medical Research Institute, Brisbane, Australia

Introduction: Androgen deprivation therapy (ADT) remains the current palliative treatment of choice for patients with prostate cancer, despite its ass ociation with multiple negative side effects. While ADT is initially effective in treating prostate cancer and metastatic prostate cancer, these cells are able to adapt such that the androgen receptor (AR) signalling axis remains active. This AR signalling continues to control cell proliferation and survival, leading to the development of castrate resistant prostate cancer (CRPC).
Methods: We first discovered that hormonal stimuli of AR expressing cells alter the protein and miRNA content of small EV. This occurs through upregulation of CD9 positive (CD9+) EV secretion, influencing the AR signalling axis. We have shown that CD9+ EV were able to drive cellular proliferation in charcoal stripped serum (CSS), indicating that EV can drive cellular proliferation and survival irrespective of the presence of androgens (1–3). Our most recent studies show that cells undergoing inhibition of AR signalling using Enzalutamide display a unique protein and miRNA profile in small EV subpopulations, which differs to that seen in CSS grown cells. Proteomic analysis of EV from Enzalutamide treated cells also does not detect multidrug resistant associated proteins, implicating unique pathways driven by EV towards CRPC progression. Assessment of CD9+ EV is relevant to advanced prostate cancer progression as our recent clinical data has found that the CD9+ EV level is significantly higher in plasma from patients with advanced metastatic prostate cancer who have detectable circulating tumour cells than in those who do not. We propose that CD9+ EV contribute towards the progression of CRPC, allowing prostate cancer cells to grow despite AR targeted therapy.
Results and Conclusion: Further targeted studies will provide a biological understanding on the role of EV in the AR signalling axis, enabling the design of novel EV based therapeutics to target CRPC.
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PS06.12

Uptake and functionality of prostate cancer extracellular vesicles depends on the metastatic stage of the parental cells

Elisa Lázaro-Ibáñez[1], Maarit Neuvonen[1,2], Maarit Takatalo[1,2], Uma Thanigai Arasu[3], Cristian Capasso[4], Johng Rhim[5], Kirsi Rilla[3], Marjo Yliperttula[1] and Pia R-M. Siljander[1,2]

[1]Division of Pharmaceutical Biosciences and Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland; [2]Division of Biochemistry and Biotechnology, Department of Biosciences, University of Helsinki; [3]Faculty of Health Sciences, School of Medicine, Institute of Biomedicine, University of Eastern Finland; [4]Laboratory of Immunovirotherapy, Division of Pharmaceutical Biosciences and Centre for Drug Research, Faculty of Pharmacy, University of Helsinki; [5]Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of Health Sciences

Introduction: Extracellular vesicles (EVs) are important mediators of cellular signalling, affecting processes such as cancer development. Internalisation of EVs can prompt functional changes in the recipient cells depending on the EV composition and origin. We hypothesised that the EVs derived from metastatic cancer cells could induce malignant properties in the recipient cells. To address this question, internalisation (uptake kinetics, effect of cell cycle) and functional effects (proliferation and migration) of EVs derived from metastatic and primary prostate cancer (PCa) cells and benign prostate cells were analysed.
Methods: EVs were isolated from LNCaP, PC-3, RC92a/hTERT and PNT2 cells by differential centrifugation at 20,000*g* for microvesicles and 110,000*g* for exosomes. Size and morphology of EVs were characterised by transmission electron microscopy and nanoparticle tracking analysis, and the presence of CD9, CD63, and HSP70 was analysed by western blotting. EVs were labelled with fixable lipophilic dyes. EV uptake was determined by high content microscopy, flow cytometry, and confocal microscopy. Cell cycle, proliferation and migration were analysed to evaluate the functional effects of the different EVs on recipient cells.
Results: EVs derived from LNCaP and PC-3 cells of metastatic origin were internalised by the recipient cells (PCa and benign) more efficiently than the EVs derived from primary cancer RC92a/hTERT cells or benign PNT2 prostate cells, as shown by flow cytometry and high content microscopy. No differences were detected in the internalisation rate of microvesicles and exosomes. Further analysis of EV uptake and cell cycle revealed higher EV numbers in the G_2/M cells than in the G_0/G_1 or S cells, indicating that the cell cycle may play a role in active EV uptake. Metastatic cell-derived EVs from PC-3 and LNCaP cells prompted more proliferative and migratory behaviour in the recipient cells (PCa and benign) compared to the EVs derived from primary cancer or benign cells.
Conclusion: These results show that the uptake and functional capacity of EVs depends on the metastatic state of the parent cells, encouraging more research into the EV-mediated mechanisms that promote tumour spread and metastasis in the tumour microenvironment.

PS06.13

Glycosylation promotes azurocidin sorting into EVs in clear cell renal cell carcinoma cells

Kentaro Jingushi[1], Takuya Naito[1], Motohide Uemura[2], Koji Ueda[3], Kazutoshi Fujita[2], Norio Nonomura[2] and Kazutake Tsujikawa[1]

[1]Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [2]Department of Urology, Osaka University, Graduate School of Medicine, Osaka, Japan; [3]Project for Personalised Cancer Medicine, Cancer Precision Medicine Centre, Japanese Foundation for Cancer Research, Japan

Introduction: We previously developed a new extracellular vesicle (EVs) isolation method, which in turn yields what we term tissue-exudative EVs (Te-EVs) from surgically resected viable clear cell renal cell carcinoma (ccRCC) tissues and adjacent normal renal tissues. LC/MS analysis revealed that azurocidin (AZU1) was enriched in ccRCC Te-EVs compared to normal renal Te-EVs ($p = 2.85 \times 10^{-3}$, fold-change = 31.59). Importantly, AZU1 was specifically detected in serum EVs from ccRCC patients but not from healthy control serum. In this study, we examined whether EV-AZU1 detected in ccRCC patient serum and ccRCC Te-EVs were derived from ccRCC cells. In addition, we searched the AZU1 sorting mechanism into EVs focused on AZU1 glycosylation.
Methods: We attempted to detect ccRCC cell-derived EV-AZU1 from AZU1-FLAG-xenografted mouse serum. PNGaseF was used for an EV deglycosylation assay. Furthermore, tunicamycin-treated ccRCC cells were analysed for western blot analysis and immunocytochemistry.
Results: AZU1 was detected in serum EVs and tumour Te-EVs obtained from AZU1-FLAG-xenografted mice. To identify the ccRCC cell-specific sorting mechanism of AZU1 into EVs, we focused on glycosylation status of EV-AZU1. An EV deglycosylation assay revealed that EV-AZU1 from ccRCC cells was enriched for N-linked oligosaccharides. Moreover, inhibition of N-linked glycosylation using tunicamycin significantly inhibited AZU1 amount on EVs in a dose dependent manner while the total particle number was not affected. Immunocytochemistry analysis revealed that tunicamycin changed AZU1 cellular localisation from golgi apparatus to throughout the cell.
Conclusion: In this study, we successfully detected EV-AZU1 from AZU1-FLAG-xenografted mouse serum, suggesting that EV-AZU1 was secreted by ccRCC cells and thus could be a potential biomarker for ccRCC. Moreover, we found that glycosylation of AZU1 is a key regulator, which in turn promotes sorting AZU1 into ccRCC-specific EVs.

Poster Session S07 – Cancer
Chairs: TBD 5:15–6:30 p.m.

LBP.47

Matrix stiffness and extracellular vesicle release

Prateek Singh and Seppo Vainio

University of Oulu, Finland

Introduction: Substrate stiffness dictate cellular function. In-vivo, cells proliferate in varying tissue mechanics, from bone hard stiffness to ultrasoft lung mucosa. Traditional biologists tend to ignore these differences. To our knowledge, this is the first study to investigate extracellular release dependence on cells cultured at varying substrate stiffness.
Methods: Mouse renal adenocarcinoma cells (RENCA) were used in this study. Cells were cultured on polydimethylsiloxane (PDMS) coated dishes. Rigidity of the culture surface was varied by using varying proportions of PDMS to catalyst ratios.
Cell viability and DNA damage were measured by Real-time GLO MT and SyTox Green cytotoxicity assay respectively. CD63 ELISA was performed using a CD63 TRIFic exosome assay. BCA assay was used to measure protein concentrations. Vesicles were separated by sequential ultracentrifugation. Briefly, cell debris was removed at 1000g, microvesicles were separated at 10,000g and extracellular vesicles were collected at 100,000g centrifugation steps.
Results: The prepared PDMS matrices represented 4 different stiffness values. The matrix stiffness for the substrates ranged from 3 gigaPascals (for polystyrene dish) to 200 kiloPascals (PDMS 40). Cell viability was highest for the stiffest substrate, polystyrene, and reduced with softer substrates. However, microvesicle yield varied less than 10% between the different substrates. Total extracellular release from the cells was within 5% variation for all but the softest, 200 kiloPascals (PDMS 40) substrate. The exosome assay however, revealed that the CD63 positive population of the extracellular vesicles, was released increasingly from more complaint matrices.
Summary/Conclusion: Our results show that cell attachment to matrix, which depends highly on the matrix rigidity, reflect the extracellular-vesicle release from the cells. This is important when comparing vesicle release from cell types originating from different tissue types, having intrinsic matrix attachment needs.

LBP.48

Characterization of saliva exosomes and exosomal microRNAs in patients with oral leukoplakia

Xiaobing Guan[1], Zachary Zhou[2], Li Chen[3], Yuanyuan Wang[4] and Jiaqi Wang[4]

[1]Beijing Stemmatological Hospital, Capital Medical University, Beijing, China; Capital Medical University School of Stomatology; [2]Columbus Academy, Ohio, USA; [3]The Research Institute at Nationwide Children's Hospital, Columbus, OH; [4]Capital Medical University School of Stomatology, Beijing, China

Introduction: Oral leukoplakia (OLK) is the most common premalignant disorder of the oral mucosa. Although histopathological analysis of biopsies showed that OLK-associated epithelial dysplasia is an important predictive factor of malignant transformation, saliva biomarkers to predict oral cancer development are lacking. Exosomes are nano-sized vesicles that are shed by producer cells and released into body fluids including saliva. Exosomes contain a complex mixture of microRNAs, mRNAs and proteins from the cell of origin, making them an ideal source for biomarker discovery and diagnostic development. Our goal was to characterize saliva exosomes and profile their microRNAs from patients with OLK, epithelial dysplasia and oral cancer.
Methods: Diagnosis of OLK, epithelial dysplasia or oral cancer was made on oral mucosal biopsies. Two ml whole-saliva from patients or normal individuals was collected, and exosomes were isolated. The concentration of exosomes was measured with Nanosight LM10 Instrument. Saliva exosomes carried cancer associated microRNAs were assessed using quantitative PCR. The expression of miR-185 was further evaluated by in situ hybridization (ISH) in oral mucosal specimens resected from patients with OLK, dysplasia or cancer.
Results: The patients with epithelial dysplasia has significantly higher concentration of saliva exosomes compared to OLK or normal control (mean 1.74 folds), while saliva exosome concentration in oral cancer patients was significantly decreased (mean 4.21 folds). The changes of exosomal microRNA levels in epithelial dysplasia or oral cancer include upregulation of miR-185, miR-200b, miR-29b, miR-409 and down regulation of miR-21, miR-320, and miR-486, with miR-185 demonstrating highest relative fold-changes in epithelial dysplasia and oral cancer compared to oral leukoplakia. ISH analysis confirmed remarkably increased expression of miR-185 in epithelial dysplasia and oral cancer patients compared to OLK patients.
Summary/Conclusion: Dynamic changes occur in saliva exosome concentration and exosomal microRNA content from epithelial dysplasia and oral cancer patients. Quantification of saliva exosome or their carried microRNAs may serve as ideal biomarkers in cancer risk assessment in oral leukoplakia patients.
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LBP.49

Metabolomic analysis of glioblastoma cell-derived extracellular vesicles

Jagdeep K. Sandhu[1], Nam H. Khieu[1], Claudie Charlebois[1], Melissa Hewitt[1] and Miroslava Cuperlovic-Culf[2]

[1]National Research Council Canada; [2]National Research Council

Introduction: Glioblastomas (GBMs) are the most common forms of malignant tumors of the central nervous system with a poor prognosis. Currently GBMs are diagnosed using magnetic resonance imaging (MRI) and validated by an invasive intracranial biopsy. The incidence of tumor recurrence and response to cancer treatment are also tracked by MRI, however, this imaging modality has numerous limitations. There remains an urgent need to develop non-invasive biomarkers for diagnostics and theranostics. GBMs release large amounts of EVs into the blood representing a rich source of biological information for biomarker discovery. The proteomic and mRNA profiles of EVs from GBMs have been studied, the metabolic profile of GBM-derived EVs is lacking, although cellular metabolomics analysis has shown distinct subtypes of GBMs.
Methods: In this study we used 3 different human GBM cell lines (U118, LN-18 and A172), isolated EVs and analyzed their metabolite content using NMR spectroscopy. GBM cells were cultured in serum-free medium for 72 h and exosomes were isolated by differential centrifugation followed by filtration. The clarified conditioned medium was concentrated and the supernatant was ultracentrifugated to pellet exosomes. GBM exosomes expressed the pan-exosome markers, CD9, CD63 and TGS101. Metabolites were extracted from parental cells, media and exosomes. 1D and 2D NMR spectra were analyzed qualitatively and quantitatively.
Results: NMR metabolomics has shown distinct profiles for cells, exosomes and media in all three cell lines. Qualitative, PCA and OPLS investigation showed over all differences in the three groups of sample sources and sample types and suggested possible metabolites of interest. Metabolite quantification using multivariate linear regression method developed in our group allowed determination of specific metabolic differences and suggested possible markers of exosomes originating from different GBM cell lines.
Summary/Conclusion: Metabolomics analysis of exosomes provides interesting markers of GBM cellular subtypes. Analysis in patients' samples is in planning stage.
Funding: National Research Council of Canada

LBP.50

Enrichment of mitochondrial proteins on tumor tissue-derived extracellular vesicles – presence in melanoma patient circulation

Su Chul Jang[1], Rossella Crescitelli[1], Johanna L. Höög[2], Valerio Belgrano[3], Roger Olofsson. Bagge[3], Karin Sundfeldt[4], Raghu Kalluri[5] and Jan Lötvall[6]

[1]Krefting Research Centre, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden; [2]Department of Chemistry and Molecular biology, University of Gothenburg, Gothenburg, Sweden; [3]Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy at University of Gothenburg, Sahlgrenska University Hospital, Gothenburg, Sweden; [4]Department of Obstetrics and Gynecology, Institute of Clinical Sciences, Sahlgrenska Cancer Center, University of Gothenburg, Gothenburg, Sweden; [5]Department of Cancer Biology, Metastasis Research Center, University of Texas MD Anderson Cancer Center, Houston, Texas, USA; [6]Krefting Research Centre, University of Gothenburg, Sweden

Introduction: EVs are attractive sources of biomarkers, because EVs that are produced from disease cells, such as cancers, can have molecular signatures of the producing cells. However, most EV-based biomarker candidates that have been identified until now are from cell culture-derived EVs and might not be valid markers for actual human disease. Here, we have isolated EVs directly from tumor tissues and analyzed the EV membrane proteome to describe biomarkers.
Methods: EVs were isolated from melanoma metastatic tissues and three cell lines, by differential centrifugation and density gradient. Membrane proteins of isolated EVs were analyzed by mass spectrometry. Through the bioinformatics analysis, biomarker candidates were selected. Selected candidates were validated both in isolated EVs and in plasma of melanoma patients by ELISA.
Results: Enrichment of mitochondrial membrane proteins was revealed in melanoma metastatic tissue-derived EVs, compared to non-melanoma-derived EVs. Further, we discovered that patients with metastatic malignant melanoma have increased concentrations of mitochondrial membrane protein containing EVs in plasma.
Summary/Conclusion: Our results show the ability of cells to release extracellular vesicles that carry multiple mitochondrial components, including several mitochondrial membrane proteins, and this rare subpopulation of EVs may be used as a novel biomarker for melanoma.
Funding: This work was funded by the Swedish Research Council (K2014-85X-22504-01-3), VBG Group Herman Krefting Foundation for Asthma and Allergy Research, the Swedish Heart and Lung Foundation (20120528), the Swedish Cancer Foundation (CAN2014/844), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1A6A3A03007377)

LBP.51

Extracellular vesicles as drug delivery vehicles for oncolytic adenovirus and paclitaxel

Mariangela Garofalo[1], Heikki Saari[1], Petter Somersalo[1], Elisa Lázaro Ibáñez[2], Laura Aksela[3], Cristian Capasso[4], Matti Jalasvuori[5], Vincenzo Cerullo[6], Paolo Ciana[7], Lukasz Kuryk[8] and Marjo Yliperttula[9]

[1]Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland; [2]University of Helsinki, Finland; [3]Orion corporation; [4]Laboratory of Immunovirotherapy, Division of Pharmaceutical Biosciences and Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland; [5]Biological and Environmental Science, University of Jyväskylä, Finland; [6]Laboratory of ImmunoVirothetherapy, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland; [7]Division of Oncology and Onco-Hematology, University of Milan (Italy); [8]Targovax ASA; [9]Division of Pharmaceutical Biosciences and Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland

Introduction: EVs are known to act as endogenous carriers of a wide range of proteins and nucleic acids, subsequently delivered to recipient cells. Hence, EVs hold great potential as being exploited as delivery vehicles of therapeutic agents. In the current study, we have investigated the EV-mediated delivery of oncolytic adenovirus and paclitaxel.
Methods: The A549 lung cancer cell line was used to produce EVs containing oncolytic adenovirus (EV-Vs) as well as control EVs. EV-Vs and EVs were incubated in 10 μM paclitaxel (PTX) solution to produce corresponding PTX complexes (EV-V-PTXs and EV-PTXs respectively). Test preparations produced were characterized using nanoparticle tracking analysis (NTA) and electron microscopy. *In vitro* virus transduction efficiency and cytotoxicity in A549 cells was assessed. Furthermore, an *in vivo* efficacy study using a lung cancer xenograft in BALB/c nude mice, was performed.
Results: A significant increase in virus transduction efficiency was observed in cells when treated with EV-Vs or EV-V-PTXs. Indeed, the viability of cells treated with EV-Vs or EV-V-PTXs was shown to be significantly lower than that observed in virus treated cells. *In vivo*, EV-V treatment was shown to control tumor growth better than virus alone.
Summary/Conclusion: In conclusion, the EV-mediated delivery of oncolytic adenovirus and paclitaxel could be a promising novel strategy for cancer targeted drug delivery.

LBP.52

Could LMWPTP be a novel player in extracellular vesicles secretion in colorectal cancer cells?

Stefano P. Clerici[1] and Carmen V. Ferreira-Halder[2]

[1]OncoBiomarkers Lab, Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, Brazil; [2]OncoBiomarkers Lab, Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, Brazil

Introduction: Phosphorylation and dephosphorylation are important processes related to post-translational modifications which influence cell signaling pathways, and when deregulated are related to diseases. Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) is upregulated in several cancers, including colorectal cancer (CRC), and it has been correlated with aggressiveness, chemoresistance and poor prognostic. Extracellular vesicles (EVs) have gained attention in cancer research due to their role in cell-to-cell communication and tumorigenesis. This study aimed to examine whether the LMWPTP could take part in EVs biogenesis and/or secretion in CRC cell lines.
Methods: HCT116 and HT29cells were purchased from BCRJ (Brazil) and then routinely grown in McCoy5A media. Western Blot (WB) was performed to analyze LMWPTP and EVs biogenesis proteins expression. For EVs isolation, cells were cultured in filtered-serum-free McCoy5A media for 12 hours. After, the EVs were isolated by ultracentrifugation (UC) and Total Exosome Isolation Reagent kit (TEI, Invitrogen). Nanoparticle tracking analysis (NTA) was performed to achieve concentration and size.
Results: WB analysis showed that LMWPTP is overexpressed in HT29 compared to HCT116 as well as EVs biogenesis related proteins LAMP-1, LAMP-2, HRS, STAM-2, Flotillin-1, Rab5, Rab7, Rab11 and Rab35 were highly expressed in HT29 *vs* HCT116; while TSG101 and ALIX were lower expressed in HT29. EVs isolated by UC and TEI methods showed that the culture media from HT29 displayed higher concentration of EVs than HCT116.
Summary/Conclusion: This study show, for the first time, that the LMWPTP might be associated to EVs biogenesis or trafficking mechanisms, once its higher expression is related to high amount of EVs from HT29 cells. Furthermore, it was observed higher expression of proteins involved in biogenesis and trafficking in HT29 compared with HCT116. These findings indicate an application potential of this enzyme in the EVs research.
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LBP.53

Characterizing the extracellular vesicle production of stromal fibroblasts in colorectal cancer

Zsuzsanna Szvicsek[1], Ádám Oszvald[1], Andrea Kelemen[1], Attila Zaránd[2], László Harsányi[2], Edit Buzás[3] and Zoltan Wiener[1]

[1]Semmelweis University, Department of Genetics, Cell and Immunobiology, Budapest, Hungary; [2]Semmelweis University, 1st Department of Surgery, Budapest, Hungary; [3]Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary

Introduction: Colorectal cancer (CRC) is the third most frequent cause of cancer-related death in Western countries. Interestingly, the expression level of a fibroblast-specific gene set shows strong correlation with patient survival in CRC. Furthermore, the activation of fibroblasts is generally considered a major factor in their tumor growth-promoting function. Here we studied whether fibroblast activating factors lead to an increased EV production and whether increased fibroblast-derived EV release may have an impact on CRC survival.

Methods: We analyzed publicly available gene expression data, normal colon- and CRC patient-derived fibroblasts. Our studies were approved by an ethics committee and informed consent was obtained. We measured the EV production of the fibroblasts by qNano as well as by bead-based methods using low and high resolution flow cytometry.
Results: Both normal colon-derived (NCF) and CRC patient-derived (CAF) fibroblasts were CD63high/CD81high/CD9low. While NCFs and CAFs secreted CD63+ and CD81+ small EVs, large Annexin V+ EVs were hardly detectable. Interestingly, major fibroblast activating molecules such as TNF alpha or TGF beta, did not increase the EV production of either NCFs or CAFs. Furthermore, bioinformatics analysis of publicly available gene expression data did not reveal a significant correlation between the expression level of genes involved in EV biogenesis and the risk of disease relapse in CRC patients. These genes also showed unchanged or decreased expression levels in CRC patients compared to normal colon samples.
Summary/Conclusion: Our data suggest that the amount of fibroblast-derived EVs may not be critical in patient survival in CRC. However, changes in their molecular content require further detailed analysis.
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LBP.54

Extracellular vesicle derived from propionibacterium acnes is a possible causative agent of prostate cancer via over-expression of androgen receptor

Sangeon Shin[1], Jin Her[2], Jinseong Jeon[2] and Changill Ban[2]

[1]POSTECH; [2]Pohang University of Science and Technology, Pohang, Republic of Korea

Introduction: Prostate cancer (PCa) is the most common non-cutaneous cancer, which is a main cause of morbidity and mortality in men in western countries. In various PCa cases, PCa patients were reported to be related with *Propionibacterium acnes* (*P. acnes*), which was human normal flora found throughout gastrointestinal tract and skin tissues.
Methods: In this study, we targeted *P. acnes*-derived extracellular vesicle (PaEV) that might cause over-proliferation of prostate cells thereby, cause prostate cancer. The PaEVs were isolated by the ultracentrifugation of *P. acnes* culture media and characterized as previously described.
Results: Through in vitro and *in vivo* studies, we confirmed immunogenicity of PaEV, and we showed that it induced the over-expression of androgen receptor (AR) which caused persistent proliferation of prostate cells. The PaEV increased the production of pro-inflammatory mediators (IL-1β, IL-6, TNF-α) by raw264.7 as dose dependent manner. After intraperitoneal injection, the PaEVs induced strong expression of AR in the prostate tissue of mice but peptidoglycan (PGN) and lipoteichoic acid (LTA) did not.
Summary/Conclusion: In conclusion, these results show the possibility that PaEVs are a novel causative agent being able to induce prostate carcinogenesis.

LBP.55

Detection and characterization of large oncosomes in thyroid cancer cell lines

Tessa Seale[1], Bonita Powell[2], Yongchun Wang[3], Dolores Di Vizio[4], Chris Umbricht[5], Martha Zeiger[6] and Kenneth Witwer[2]

[1]The Johns Hopkins School of Medicine, the Graduate Training Program in Cellular and Molecular Medicine, MD, USA; [2]The Johns Hopkins University School of Medicine, MD, USA; [3]The Johns Hopkins School of Medicine, Department of Surgery, MD, USA; [4]Cedars Sinai Medical Center, CA, USA; [5]The Johns Hopkins School of Medicine, Department of Surgery, Department of Oncology, MD, USA; [6]The Johns Hopkins School of Medicine, Department of Surgery, Department of Oncology, MD, USA

Introduction: Tumor invasion and metastasis can be mediated by the distribution of tumor-derived extracellular vesicles, which carry oncogenic material. Tumor cells can also undergo non-apoptotic membrane blebbing to form large extracellular vesicles (EVs) known as large oncosomes (LOs) that are between 1 to 10 μm in diameter. Amoeboid phenotype associated with LO formation can be induced with Epidermal Growth Factor (EGF) treatment and knockdown of DIAPH3, an actin-nucleating protein. LO formation has not yet been described in thyroid cancer.
Methods: Anaplastic thyroid cancer (ATC) and papillary thyroid cancer (PTC) cell lines (TPC-1, BCPAP, C643, SW1736) were used to study LO formation. PTC and ATC lines were cultured with no treatment, treated with epidermal growth factor (EGF), or subjected to DIAPH3 knockdown using siRNA. Cells and LOs were stained with Cholera Toxin subunit B to visualize the membrane using fluorescence microscopy. LOs were also measured by flow cytometry (LSR Fortessa). LOs were separated from supernatant components by low-speed centrifugation or by a centrifugation and filtration strategy, and RNA was isolated from LOs and cells for miRNA profiling using custom TaqMan low density arrays.
Results: LO formation was detected in four thyroid cancer cell lines using both fluorescence microscopy and flow cytometry. Treatment of EGF caused an increase in the amoeboid phenotype and LO production in all four cell lines, with a more striking change in phenotype occurring in the PTC lines. Moreover, knockdown of DIAPH3 increased LO formation in all lines.
Summary/Conclusion: LO production in thyroid cancer cell lines can be detected using microscopy and flow cytometry. Treatment of EGF and DIAPH3 knockdown both resulted in an increased amoeboid phenotype, implying that thyroid cancer LOs form in a manner similar to previously studied LOs in prostate cancer. Future directions include identifying quantitative differences of LO production between the different thyroid cancer cell lines as well as characterizing the protein and RNA content within the LOs.

LBP.56

A potential exosome biomarker for non-small cell lung cancer by proteomics analysis

Hyesun Jeong[1], Byeonghyeon Choi[2], Jik Han Jung[3], Jaena Park[4], Yong Park[5], Ji Ho Park[3], Yeonho Choi[6], Hyun Koo Kim[7] and Sunghoi Hong[8]

[1]Korea University Department of public health, Korea University, Seoul 136-701, Republic of Korea; [2]Korea University, Seoul, Republic of Korea; [3]KAIST, Seoul, Republic of Korea; [4]Korea University, Seoul, Republic of Korea; [5]Division of Hematology-Oncology, Department of Internal medicine, Korea University Anam Hospital, Korea University College of Medicina, Seoul, Republic of Korea; [6]Department of Bioconvergence Engineering, Korea University, Seoul, Republic of Korea; [7]Department of Thoracic and Cardiovascular Surgery, Korea University Guro Hospital, Korea University College of Medicine, Republic of Korea; [8]School of Biosystem and Biomedical Science, Korea University, Seoul, Republic of Korea

Introduction: Exosomes are cell-derived vesicles, which are ranged from 50 to 150 nm size, that are secreted in perhaps all eukaryotic fluids, such as blood, urine and cell culture medium. Since they have specialized functions and play a role in many biological processes such as intercellular signaling, there is a growing interest in the clinical applications of exosomes such as diagnostic biomarkers for cancer.
Methods: Exosomes from Non-small cell lung cancer (NSCLC) cells and Human Pulmonary Artery Endothelial Cell (HPAEC) were isolated by column liquid chromatography and analyzed by Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA) and western-blotting (CD63). The exosomes were lysed and applied to proteomic analysis.
Results: Five proteins were identified in NSCLC exosomes but not HPAEC. One of them was dramatically increased in NSCLC cell lines- and NSCLC patients-derived exosomes but not normal HPAEC by our quantitative RT-PCR and western blot. The protein was named as lung cancer exosome-specific protein 1 (LESP1), which is involved in endosome-to-Golgi transport. Summary/Conclusion: The protein, LESP1, may be a potential biomarker for NSCLC diagnosis. Funding: This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HR14C0007).

LBP.57

Comparative analysis of EV gene products to subcellular fractions in a K-562 human lymphoblast cell model

Fabio Alexis Lefebvre[1], Juan-Carlos A. Padilla[2], Neal Cody[3], Louis Philip Benoit Bouvrette[1], Janusz Rak[4] and Eric Lécuyer[5]

[1]Institut de Recherche Clinique de Montréal (IRCM), Montréal, QC, Canada; Département de Biochimie, Université de Montréal, Montréal, QC, Canada; [2]Institut de Recherches Clinique de Montréal (IRCM), Montréal, QC, Canada; Division of Experimental Medicine, McGill University, Montréal, QC, Canada; [3]Icahn School of Medicine, Mount Sinai, New York, NY, USA; [4]Montreal Children's Hospital, Research Institute of the McGill University Health Center, Montreal, QC, Canada; [5]Institut de Recherches Clinique de Montréal (IRCM), Montréal, QC, Canada; Département de Biochimie, Université de Montréal, Montréal, QC, Canada; Division of Experimental Medicine, McGill University, Montréal, QC, Canada

Background: Efforts in the study of extracellular vesicles (EVs) have revealed diverse species of gene products being shuttled out of many cell models. Although much effort has been placed in the characterization of EVs contents, less attention has been given to investigating the subcellular targeting of gene products to EVs. Here, we report a large-scale comparative analysis of protein and RNA contents in different subcellular fractions versus those found in EVs.

Methods: EVs were isolated from human K-562 lymphoblast cells by differential centrifugation of cell culture media at 110,000 × *g* for 60 minutes. Subcellular fractions were isolated from pelleted cells using a validated biochemical approach with the use of a sucrose cushion technique to isolated nuclear and cytosolic fractions, and Titron-X to separate membrane and insoluble fractions. The isolated fractions and EVs were subject to proteomic profiling using liquid chromatography-tandem mass spectrometry (LC-MS/MS), while RNA distribution was analysed through deep sequencing of long and short RNAs.

Results: Out of 3355 identified proteins, only 31 were ubiquitous across all studied fractions, suggesting that there is a strong spatial asymmetry in the distribution of these proteins. On the other hand, pairwise correlative analysis of Exponentially Modified Protein Abundance Index (% emPAI) values revealed linear and ordinal associations among the proteomic signatures of EVs and the cytosolic fraction. RNA distribution analysis showed that transcripts found in EVs were poorly expressed in total cell extracts (Kruskal-Wallis test; $P \leq 10^{-4}$), while exhibiting distinctive functional signatures. Analysis of the cytotopic distribution of small regulatory RNAs revealed that read length distributions were distinctive and reproducible across fractions, and similarities in the content of EVs and the cytosolic fraction were observed.

Summary/Conclusion: Our comparative analysis point to a semi-selective model of targeting and incorporation of gene products into EVs, which is highlighted by the spatial asymmetry of protein distribution among subcellular fractions, and the association of proteomic and RNA signatures between EVs and the cytosolic fraction.

Poster Session S08 – Viruses, Bacteria, Fungi, and Parasites
Chairs: Vincent Bond and Linda Coughlan 5:15–6:30 p.m.

LBP.58

Characterization of extracellular vesicle (EV) concentration and size distribution following pathogen inactivation treatment of platelet components

Paula Saá[1], Tracey Díaz[2], Felicia Santa María[2], Anoop Pal[3], Gary Holley[1], David Krysztof[1], Adonis Stassinopoulos[2] and Susan Stramer[1]

[1]American Red Cross; [2]Cerus Corporation; [3]Izon Science

Introduction: New strategies have been developed to inactivate infectious agents in blood. Cerus' INTERCEPT™ Blood System for platelet components combines amotosalen with Ultraviolet (UVA) illumination to specifically target nucleic acids and irreversibly cross-link or form adducts on these molecules, thereby blocking pathogen replication. The efficacy and safety of amotosalen/UVA treated platelet and plasma components has been broadly investigated, the effects on EVs and their biological activity have not been extensively explored.
Methods: Apheresis platelet concentrates (PCs) in 100% plasma were collected from healthy donors and pathogen inactivated following standard procedures. To evaluate changes in EV size and concentration, a control sample aliquot was removed from the PCs after amotosalen addition, but before UVA illumination. PCs were then illuminated and a post-treatment aliquot collected. EVs were isolated from pre- and post-illumination samples by centrifugation and size exclusion chromatography (SEC) and their presence was assessed by biochemical and biophysical methods.
Results: EVs were detected by electron microscopy imaging in all samples regardless of treatment. The size and morphology of these vesicles (30-50 nm) was consistent with literature reports. No substantial qualitative changes were observed between pre- and post-illumination samples. Comprehensive analyses of pooled SEC fractions 9-11 by tunable resistive pulse sensing confirmed the absence of statistically significant differences in EV concentration (3.39E^10 vs. 3.81E^10) and size (157 vs. 158 nm) between pre- and post-illumination samples, respectively. Likewise, protein analysis of EV lysates by quantitative and qualitative assays did not find major phenotypic changes.
Summary/Conclusion: These results suggest that INTERCEPT does not induce significant changes in EV size, concentration and phenotype. Additional studies will evaluate their biological properties and clinical applications.
Funding: Cerus Corporation and American Red Cross.

LBP.59

Exosome involvement in JC Polyomavirus Infection

Aisling S. Dugan[1], Bethany O'Hara[2], Gretchen Gee[2], Benedetta Assetta[2], Jenna Morris-Love[3] and Sheila Haley[2]

[1]Assumption College, MA, USA; [2]Brown University, RI, USA; [3]Pathobiology Graduate Program, Brown University, RI, USA

Introduction: Human JC polyomavirus (JCPyV) causes the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML) in AIDS patients. JCPyV utilizes the sialyated glucan, LSTc, and the serotonin 2 subgroup of receptors to gain entry into the human glial cell line, SVG cells. Paradoxically, patient isolates of JCPyV from PML patients have mutations in the major viral capsid protein, VP1, that prevent binding to the serotonin receptor and infection of SVG cells. Moreover, some primary cells without the LSTc receptor can be infected with JCPyV. These observations suggest that there may be an alternative route for JCPyV infection in humans that does not involve the canonical receptors. Exosomes are small (30-100 nm) vesicles released by cells shown to be critical for cell-cell communication and important in the spread of some viruses.
Methods: Exosomes were isolated from JCpyV infected SVG cells and examined for exosome number, infectivity, and visualized using transmission electron microscopy.
Results: Our data showed that exosomes isolated and purified from the supernatant of JCPyV infected SVG cells contain VP1 and are infectious. JCPyV infection increased the number of exosomes released in media compared to uninfected cells. Exosome inhibitors block JCPyV spread. Transmission electron microscopy revealed that exosomes isolated from JCPyV infected cells were found within vesicle-like sacs consistent with exosomes.
Summary/Conclusion: Together, these data suggest a role for exosomes in the spread of infectious JCPyV.
Funding: NIH funded: 2R01NS043097-15A1.

LBP.60

Extracellular vesicles in the immune response to Hepatitis-Virus infections

Stephanie Jung[1], Yuchen Xia[1], Michaela Gack[2] and Ulrike Protzer[1]

[1]Helmholtz Center Munich, Munich, Germany; [2]University of Chicago, IL, USA

Introduction: Hepatitis B Virus (HBV) infection is a global health issue with 240 million chronical carriers and a major driver of liver cirrhosis and hepatocellular carcinoma. Coinfection of HBV-positive patients with Hepatitis D Virus (HDV) enhances the risk of severe fulminante hepatitis with liver failure and high mortality rates. Current therapies against HBV prevent the progression of the disease but are ineffective in mediating viral clearance. We aim to understand the immunomodulatory role of EVs in HBV and HDV infection. HBV is considered a stealth virus, fundamentally blocking cytokine production in the infected cell by extensive immune evasive mechanisms. HDV is a satellite virus that requires HBV for virus production and propagation.
Methods: We hypothesize that HBV/HDV infected cells shuttle exosomes to non-infected cells, herewith modulating the innate immune response. To test this hypothesis, we purify and transfer exosomes produced by virus-infected cells to non-infected immune cells and quantify cytokine production by both qRT-PCR and ELISA.
Results: Preliminary results indicate an immuno-modulatory effect of exosomes released by HDV-infected cells. Additionally, we observe that both intracellular HBV-DNA and HBV transcription levels are diminished in response to transfer of supernatant derived from IFN-α pretreated cells. It can be shown that not interferon itself but heparin-binding particles of high molecular weight released by pretreated cells are responsible for this effect. These very particles inhibit virus entry into hepatoma cells and interact with the HBV receptor heparan glycosaminoglycan.
Summary/Conclusion: Resulting data shall elucidate mechanisms of HBV and/or HDV pattern recognition by the immune response. Not only the mode of signal transmission, but also detected pathogen-associated molecular patterns and their corresponding receptors can be identified. These results might give insight into additional HBV-detecting pattern recognition receptors.
Funding: SJ is funded by the Helmholtz Association's Initiative and Networking Fund, YX is partly sponsored by The International Liver Cancer Association and MG is funded by NIH.

LBP.61

How much exosomes will mimic physiological response in *in vitro* experiment? Learning from Extracellular vesicles mediate signaling in ocular system

Elie Beit-Yannai, Sofia Schreiber-Avissar and Natalie Lener

Ben-Gurion University, Israel

Introduction: Extracellular vesicles (EVs) mediated signaling attract researcher in many biological disciplines, and many research are conducted in-vitro. How much EVs are required to mimic the physiological condition is unclear. EVs calibrated according to their protein content were used in the range of 1μg to 50μg per couple of millions targeted cells. In most of the cases

EVs dose response was not addressed. In the present research we examine the effects of different concentrations of EVs derived from the aqueous humor producing cells (NPCE) on the trabecular meshwork (TM) cells. Communication between these tissues *in-vivo* is considered important for maintaining the intra ocular pressure and have an important role in glaucoma disease. Changes in gene, protein expression and activity of the Wnt signaling pathway members, known to be involved in the pathology of glaucoma disease, were examined according the tested EVs doses.
Methods: Human NPCE cell line was grown in exosome depleted medium and EVs were extracted using the PEG precipitation method followed by ultra-centrifugation. The exosomes were incubated for 8h, at three different concentrations ($6.8*10^9$ (X1), $13.6*10^9$ (X2) or $60.8*10^9$ (X10) with $0.5*10^6$ TM cells. EVs were determined using Bradford and FRAP methods. Retinal pigment epithelium cells derived EVs were used as control and incubated at the same concentrations with $0.5*10^6$ TM cells. Quantitative PCR, Western Blot analysis and Zymography were used.
Results: Exposure of confluent TM cells for 8h to various concentrations of EVs, lead to significant changes in the expression of the measured proteins. Exposure to low exosome concentration was associated with decreased expression of β-Catenin, GSK-3β, as compared to exposure to high exosomal concentrations ($p < 0.01$). When exosomes were incubated with TM cells for 2h, a tendency of decrease was found in β-catenin, Axin2 and LEF1 mRNA levels in low concentrations of exosomes compared to high ones.
Summary/Conclusion: Cross talk between the ocular drainage tissues via EVs exist . NPCE derived EVs specifically target the TM cells resulting in changes in the TM ECM
Our findings suggest that EVs concentration plays a major role in the NPCE-TM communication in-vitro. Furthermore, a bimodal TM response to exosome concentration exposure was found.

LBP.62

Blood neuron-derived exosomes as biomarkers of cognitive impairment in HIV infection

Lynn Pulliam[1], Bing Sun[2] and Pranjali Dalvi[3]

[1]University of California, San Francisco, CA, USA; [2]Veterans Affairs Medical Center, USA; [3]Veterans Affairs Medical Center, USA

Introduction: A subset of HIV-infected subjects continues to have cognitive impairment in spite of effective therapy suppressing viral load. This may be due to the high probability of a persistent viral reservoir and ongoing injury. Finding an inexpensive, noninvasive, peripheral biomarker for cognitive impairment has been a high priority. Exosomes (exos) are shed from most cells including neural cells. We isolated neuron-derived exosomes (NDE) in plasma from controls and HIV-infected subjects with varying degrees of cognitive impairment.
We examined these NDE for markers of neuronal damage.
Methods: Total exos were isolated from plasma of 12 healthy control subjects and 23 HIV-infected subjects using Exoquick. Institutional informed consent was obtained from all subjects. All HIV positive subjects were on antiretroviral therapy with none to varying degrees of cognitive impairment; most had controlled viral load. Further isolation to NDE was performed by immunoadsorption using antibody to the neuron specific cell marker L1CAM. NDE were characterized by NSE, NF-L and synaptophysin (SYP). We looked at exo numbers, CD81 and protein content by ELISA for 2 targets associated with neurodegeneration, Aβ and HMGB1.
Results: HIV-infected subjects had significantly fewer total plasma exos compared to controls but there was no difference in NDE numbers; NF-L and SYP were elevated in NDE. Neuropsychologically impaired (NPI) subjects had significantly fewer NDE. HMGB1, Aβ and NF-L were increased in NDE from impaired compared to normal subjects.
Summary/Conclusion: We demonstrate for the first time that the number of total exos in the plasma of individuals with HIV infection is decreased. Subjects with cognitive impairment had decreased NDE suggesting fewer neurons. However, within NDE, there is a significant increase in several targets including an alarmin, HMGB1, from impaired subjects. NDE cargo reflects neuronal damage and may predict a cognitive impairment profile.
Funding: National Institutes of Health, MH085538 and MH112483 (to L.P.).

LBP.63

Withdrawn at author's request.

LBP.64

Fish MVs: A diagnostic tool?

Leidy Lagos[1], Sabina Leanti La Rosa[2], Julia Tandberg[3], Hanne Winther-Larsen[3] and Margareth Øverland[2]

[1]Norwegian University of Life Sciences, Oslo, Norway; [2]University of Life Sciences, Oslo, Norway; [3]University of Oslo, Oslo, Norway

Introduction: Extracellular vesicles (EVs) with lipid bilayers consist of apoptotic bodies, ectosomes, microparticles, microvesicles and exosomes. Exosomes represent endosome-originated small membrane vesicles, about 30–100 nm in diameter that are secreted by cells and contain several molecules, including proteins and nucleic acids. The secretion of MVs is a common feature of most cells, including eukaryotic cells and bacteria. *Piscirickettisa salmonis* is a facultative intracellular pathogen that affects farmed salmonids around the world. This Gram-negative fastidious bacteria produces a systemic infection characterized by colonization of several organs in the fish and is able to infect, survive, and replicate inside macrophages, however little is known about its mechanisms of pathogenesis. Recently, we have proven that *P. salmonis* is able to secrete MVs as strategies to deliver virulence factors to the eukaryotic host cells.
Methods: MVs were isolated from salmon (*Atlantic salmon*) plasma that were infected with the fish pathogen *P. salmonis* using a commercial kit and their presence was confirmed by transmission electron microscopy, Nanosizer, flow cytometry and its protein content was analyze by mass spectrometry.
Results: The proteomics results shown 113 protein present in both, healthy and infected fish. However, 106 proteins were present just in the group infected with *P. salmonis*. Among these proteins are heat shock protein 90, proteasomes subunit, complement protein, autophagy and major histocompatibility class (MHCI), which are key to overcome intracellular infection. Interestingly, we were able to identify bacterial proteins, such as phenylalanine–tRNA ligase, porin and diguanylate cyclase in the plasma of infected animals. Our results suggest that the protein present in MVs depend of the health status of the animal, as well as confirming the ability of *P. salmonis* to secrete MVs *in vivo*.
Summary/Conclusion: Our results suggest that the protein present in MVs depend of the health status of the animal, as well as confirming the ability of *P. salmonis* to secrete MVs *in vivo*.

LBP.65

Extracellular vesicle-associated miR-155 may contribute to HIV-1 pathogenesis

Audrey Hubert[1], Caroline Subra[2], Julien Vitry[1], Myriam Vaillancourt[1], Anne Bergeron[1], Frédéric Barabé[1], Mohammad-Ali Jenabian[3], Cecile Tremblay[4], Jean-Pierre Routy[5], Provost Patrick[6], Sylvie Trottier[1] and Gilbert Caroline[6]

[1]Université Laval, Quebec, Canada; [2]U.S. Military HIV Research Program; [3]UQAM; [4]CHUM; [5]McGill University, Montreal, Canada; [6]Department of Microbiology-Infectious Disease and Immunity and Faculty of Medicine, Université Laval, Quebec, Canada

Introduction: Extracellular vesicles (EVs) mediate cellular communications and transformation, and contain genetic materials. The abundance of EVs in plasma (pEVs), and their microRNA content may thus be used as biomarkers of inflammatory diseases such as HIV infection. We reported that pEVs isolated from antiretroviral-therapy-naïve HIV-1 patients are enriched in miR-155. However, the type of microRNA-containing EVs and their relevance to HIV-1 pathogenesis remains unknown.

Methods: pEVs were precipitated from plasma ART-treated and untreated patients, elite controllers and healthy individuals (n=8/group) by using ExoQuick™ and separated by velocity gradients. Selected microRNAs were detected by qPCR, and the impacts of EVs enriched in miR-155 were tested *in vitro* and *in vivo* by using relevant HIV-1 infection models.
Results: We observed an increased abundance of acetylcholinesterase-positive pEV (exosomes) in first fractions, and concentrations of EV-borne miR-155 in mischaracterized denser fractions in the case of ART-naïve subjects. Peripheral blood mononuclear cells or NOD/Scid/IL2rγnull (NSG) humanized mice responded to miR-155-bearing vesicles with a marked decrease in the CD4+/CD8+ T lymphocyte ratio resulting from an increase in CD8 T cells, and with the expression of exhaustion marker PD-1 and increased viral production.
Summary/Conclusion: This study confirms that the pEV population increases in heterogeneity during infection with HIV-1 and those ART-naïve patients appear to have uncharacterized pEVs that are larger than exosomes and enriched in miR-155. This study showed that velocity gradient remains the most effective method of resolving the pEV population. More importantly, we provide evidence that miR-155-enriched EVs affect HIV-1-associated pathogenesis by promoting activation of CD8 T cells and possibly exhaustion on the long term.
Funding: This study was funded through grants MOP-267056 (HIV/AIDS initiative) to C.G., a FRQ-S AIDS and infectious Diseases Network grant to C.G. and S.T, grant MOP-03230 to J.P.R. and C.T. (for cohort establishment) and by the FRQ-S AIDS and infectious Diseases Network. This work was supported in part from a grant awarded to Drs Barabé and Gilbert through a donation of Merck Sharpe & Dohme Corp. to the Faculty of Medicine via the Fondation de l'Université Laval.

LBP.66

Role of Nedd4-family members in assembly and release of quasi-enveloped hepatitis A virus (eHAV)

Olga González-López[1], Kevin L. McKnight[1] and Stanley M. Lemon[2]

[1]University of North Carolina, NC, USA; [2]University of North Carolina at Chapel Hill, NC, USA

Hepatitis A virus (HAV) is an important cause of enterically transmitted hepatitis in humans and a unique picornavirus that is released from cells non-lytically enclosed in 50-110 nm membranous vesicles. These infectious, quasi-enveloped virions (eHAV) are the only form of virus found in blood from infected humans. Release of eHAV from infected Huh-7.5 hepatoma cells is ablated by siRNA knockdown of ALIX or VPS4B, ESCRT-associated proteins required for exosome biogenesis via the multi-vesicular body (MVB) pathway. Consistent with this, a proteomics analysis of gradient-purified eHAV identified numerous host proteins normally localizing to endolysosomes. Among these, NDFIP2 (Nedd4-family interacting protein 2) is an adaptor protein that binds and activates HECT domain Nedd4 family E3 ubiquitin ligases and that has been implicated in sorting of cargo for delivery to MVBs.

LBP.67

Blueberry fruit nano vesicles

Ryan Yu and Benjamin D. Zeitlin

University of the Pacific Arthur A. Dugoni School of Dentistry, CA, USA

Introduction: Nanovesicles have been identified in preparations of edible plants and certain fruit although the exact cellular origin of these vesicles remains unclear. Cross-kingdom biological effects have been demonstrated for nanovesicles isolated from certain fruit - notably lemons and grapes - that are absent from normal, dietary intake of the whole fruit, this suggests a therapeutic potential for isolated fruit vesicles. Anthocyanins and other polyphenolic anti-oxidant compounds are known to accumulate in plant vesicles as are specific proteins, lipids and microRNAs species. Here we investigate the presence of nanovesicles in antioxidant-rich blueberry fruit.
Methods: Fresh blueberries were manually crushed and the pulp passed through a course sieve. Pulp was diluted with phosphate buffered saline and subject to differential centrifugation and ultracentrifugation. The resulting pellet was insoluble and highly resistant to disruption. A jelly-like consistency of the pellet suggested precipitation of soluble structural polysaccharide such as pectin common to soft fruits. To examine the pellet, a sample was fixed in formaldehyde/glutaraldehyde, dehydrated in acetone and embedded in resin. The sample was sectioned and subject to transmission electron microscopy (TEM).
Results: We observed sections with numerous vesicle-like structures - approximately 30-100 nm - in addition to highly fibrous areas but generally not in the same field.
Summary/Conclusion: In summary we report we believe for the first time the presence of nanovesicle-like structures in extracts from fresh blueberries. We also highlight a previously unreported challenge to vesicle isolation from berry fruit in the form of a fibrous matrix.
Funding: University of the Pacific Dugoni School of Dentistry intramural funds.

LBP.68

Withdrawn at author's request.

LBP.69

Characterization of extracellular vesicles released from parasitic nematodes with different host adaptation

Eline Palm Hansen[1], Kasper Lind Andersen[1], Antonio Marcilla[2], Aaron Jex[3], Robin Gasser[3], Neil David Young[3], Ross Stephen Hall[3], Merete Fredholm[1], Stig Milan Thamsborg[1] and Peter Nejsum[4]

[1]University of Copenhagen, Denmark; [2]Universitat de Valencia, Valencia, Spain; [3]University of Melbourne, Melbourne, Australia; [4]Aarhus University, Denmark

Introduction: Parasitic worms have developed an amazing ability to evade or modulate the host immune response and recent studies indicate that extracellular vesicles (EVs) are crucial for host-parasite interactions. In this study, we examined EVs released from three gastrointestinal nematodes of pigs, *Ascaris suum, Trichuris suis* and *Oesophagostomum dentatum*. These parasites have different patterns of migration and location in the host, as well as the immunological response they evoke. They could therefore represent suitable candidates to explore unique as well as common modes of host adaptation and immune modulation.
Methods: Adult worms were incubated in RPMI under sterile conditions and EVs were purified by differential centrifugations, including two ultracentrifugations at 110,000 x g, and identified by transmission electron microscopy. EV RNA was isolated and sequenced, and the miRNAs for each of the three species were identified using miRDeep2. Predicting miRNA targets and potential functions is part of an on-going analysis, but preliminary results indicate immune-related properties of highly expressed miRNAs. In order to visualize the uptake of EVs and the subsequent transfer of EV RNA into host cells, EVs were treated with RNase, labelled with EV membrane stain as well as RNA stain, transferred to intestinal epithelial cells (Caco-2) and examined by confocal microscopy at 37°C.
Results: We found that EVs of all three species were taken up by intestinal epithelial cells followed by a release of RNA, which had a tendency to accumulate in the cell nuclei. We are currently undertaking *in vitro* studies to functionally test the role of EVs on immune cells.
Summary/Conclusion: The findings of this study contribute to unraveling the complex interplay between parasites and their hosts, which may provide new targets for diagnostic tests and novel ways for intervention and disease control in the future.
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LBP.70

Heligmosomoides polygyrus vesicle-derived small RNAs inside mouse cells: detection and targets

Jose Roberto. Bermúdez[1], Franklin W.N. Chow[2], Amy H. Buck[3] and Cei Abreu-Goodger[4]

[1]LANGEBIO, CINVESTAV, IRAPUATO MX; [2]Institute of Immunology and Infection Research, Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom; [3]Institute of Immunology and Infection Research, Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, United Kingdom; [4]Langebio–Cinvestav

Introduction: Evidence for a cell-to-cell communication system mediated by vesicles loaded with small RNAs (sRNAs) is accumulating. However, we still ignore if sRNA-loaded vesicles could be used for communication between different species. *Heligmosomoides polygyrus* is a nematode that infects mice and is a valuable model to study the immune response to chronic infections. During its adult phase, *H. polygyrus* secretes vesicles while residing in the mouse intestine. We have already shown that these vesicles have immune-modulation capacity, and that they contain proteins as well as small RNAs. We ultimately aim to determine if these *H. polygyrus* small RNAs (Hp-sRNAs) have a role during interaction with the host. Nematode vesicles have the capacity to enter MODE-K epithelial intestine mouse cells *in vitro*, as revealed by fluorescently labelling the nematode vesicles.

Methods: To explore in an unbiased manner the capacity of Hp-sRNAs to enter mouse cells we cultured mouse cells together with *H. polygyrus* vesicles or total secretion and performed Small RNA sequencing (sRNA-Seq). To determine if there is specificity in Hp-sRNAs internalization, we used two different cell types: MODE-K and bone marrow derived macrophages. To assess the stability of Hp-sRNAs inside mouse cells we applied two incubation times (4 and 24 hours), prior to sequencing.

Results: We can use bioinformatics to disentangle nematode and mouse sRNAs with mapping strategies and differential expression analyses. We identify 75 high confidence Hp-sRNAs inside MODE-K cells after treatment. These sRNAs are found in vesicles and tend to belong to the 22G siRNA class (nematode specific). We are currently exploring the targeting capacity for these Hp-sRNAs in mouse, and looking for over-represented functions among putative targets.

Summary/Conclusion: Our project explores the possibility of sRNA-loaded vesicles being used as a means for inter-species communication.

Industry Poster Session

IP.01

Direct from sample surface marker based single exosome counting and characterization

George Daaboul[1], David Freedman[2], Mani Sadredini[3], Steven Scherr[4], Paola Gagni[5], Marcella Chiari[5], John Connor[6] and Selim Unlu[7]

[1]nanoView Diagnostics Inc.; [2]nanoView Diagnostics; [3]Oslo University Oslo, Norway; [4]Boston University College of Mechanical Engineering, MA, USA; [5]Consiglio Nazionale delle Ricerche, Istituto di Chimica del Riconoscimento Molecolare; [6]Boston University School of Medicine, MA, USA; Department of Microbiology; [7]Boston University College of Electrical and Computer Engineering, MA, USA

Introduction: Exosomes are currently characterized by running non-specific nanoparticle analysis followed by proteomic analysis to confirm the existence of exosome markers. This approach usually requires exosomes to be isolated through ultracentrifugation (UC) to separate interference from soluble markers in the biological fluid. Recently, flow cytometers have been adapted to combine light scatter measurements from nanoparticles with fluorescent detection of exosome markers. The combination of the light scatter with specific markers improves the reliability and specificity of exosome detection. However, the small-size of exosomes makes specific detection above background levels difficult because these diameters (50-200 nm) are too small for traditional visualization technologies.
Methods: We have applied a label-free microarray imaging technique for enumeration, sizing and phenotyping of exosomes. The technique is termed Single Particle Interferometric Reflectance Imaging Sensor (SPIRIS) that allows visualization of individual nanovesicles captured on the sensor surface, which is functionalized with a non-fouling polymer arrayed with antibodies against surface markers. The sensor is comprised of a silicon substrate with a thin silicon dioxide layer forming a common path interferometer. The spectrally reflected light from the sensor surface interferes with the scattered light from captured nanoparticles enhancing their visibility.
Results: We have verified the sizing sensitivity of the sensor using viral particles from cell culture media spanning diameters from 40 nm (Zika Virus) to 360 nm (Vaccinia Virus). We have also demonstrated direct-from-sample extracellular vesicle phenotyping from cell culture media and human plasma. To validate specificity of direct-from-sample detection, results were compared to detection post-isolation using UC.
Summary/Conclusion: A detection limit of 5 x 10^5 particles/ml or 0.5 zepto moles when using 5 µl of sample was demonstrated. Direct detection of exosomes from cell culture and human plasma is shown without the need for isolation. SPIRIS direct-from-sample high-throughput technique could improve standardization of exosome preparations and facilitate translation of exosome-based liquid biopsies.

IP.02 (Gold Sponsor Abstract)

Development of an integrated methodology for extracellular vesicle purification, characterization and linking biophysical properties to biological function

Anoop Pal, Robert Vogel, Julien Muzard and Murray Broom

Izon Science

Introduction: Extracellular Vesicles (EVs) are heterogeneous in size, number, membrane composition and contents. A thorough understanding of this diversity and the linkage of biophysical properties to EV biological role and function is necessary. Real, validated, repeatable measurement data are required for the biomedical adoption of EV based diagnostics and therapeutic developments. These have not always been prominent in EV research. We also believe that normalization of any biochemical analyses back to the EV particle properties will become a standard requirement.
Methods: An integrated, standardizable and very practical methodology for EV purification and measurement has been developed. SEC columns provide clean EVs from biological fluids and cell culture, 99% free of non-vesicular proteins. TRPS provides detailed, calibrated measurement of EV particle number, size, concentration of each size fraction, and individual particle surface charge. Continual improvements by users in the usability and reproducibility of TRPS have reduced the time required while improving quality.
Results: In the first case study, EVs purified from BAL fluid were quantified and analyzed for difference in size, concentration over a defined size range and surface charge post lung exposure to nanoparticles. In second case study tissue factor bearing microparticles from two different cell lines were separated, characterized, and functionally evaluated by tissue factor activity assay. The assay responses were normalized to the TRPS data to assess the impact of particle size, surface area and volume on tissue factor activity. Further, quantification of EV surface markers (CD63 and CD142) and phenotyping of specific EVs captured via antibody conjugated to magnetic beads was achieved. Our results showed a proportional increase in size, volume and surface charge of the EV-Magnetic bead complex (immunoprecipitated) over a defined dose-range. Secondary measurements confirmed these findings as well.
Summary/Conclusion: Thus, the proposed integrated methodology provides a simple, rapid, reliable, and cost efficient approach for EV purification and biophysical characterization amenable for diagnostic and therapeutic proposes.

IP.03

Particle Size and refractive index derived from three-dimensional light scatter data

Oliver Kenyon

Apogee Flow Systems Ltd

Introduction: The complex relationship between particle size and the amount of light scattered at different collection angles makes it difficult to infer particle size from a flow cytometer's light scatter data. A population may be described as scattering an amount of light equal to a reference particle (e.g. a latex or silica bead of known size) but same sized particles of different refractive index give different signal strengths. When comparing data between flow cytometers the difficulties are compounded by differences in light scatter illumination and collection angles
Methods: A particle suspension containing a continuum of particle sizes of well-defined and known refractive index may be used to characterize the light scatter optics of any flow cytometer. Once the light scatter optics have been characterized in this way, data from biological samples (e.g. virions, extracellular vesicles) can be transformed from light scatter space (e.g. small, medium and large angle dimensions) to size and refractive index dimensions.
Results: It is possible to convert light scatter data into particle size and refractive index information. This may be thought of as a conversion from three (or more) dimensional light scatter space to 2-dimensional space with dimensions 'size' and 'refractive index'.
Summary/Conclusion: Size and refractive index parameters allow comparison of data among flow cytometers and other particle analyzers in a way not possible with light scatter data. For this reason it is well suited to studies of submicron particles such as bacteria, virus and extracellular vesicles. The new size and refractive index parameters can be stored in FCS format, compatible with widely available software.
Funding: Apogee Flow Systems Ltd

IP.04

Application of liposomes for study of biological microvesicles

Oleg Guryev[1], Tatyana Chernenko[1], Majid Mehrpouyan[1], Gulam Shaikh[1], Pam Canaday[2], Claudia Lopez[3], Terry K. Morgan[4] and Marybeth Sharkey[1]

[1]BD Biosciences; [2]Department of Pathology, OHSU; [3]Multiscale Microscopy Core, Center for Spatial Systems Biomedicine, OHSU; [4]OHSU

Introduction: Liposomes are nano/micro-size sphere-shaped lipid vesicles of single or multiple lipid bilayers. They are often used as a model objects in study of biological microvesicles (MVs). However, there is no standardized method for preparation of liposomes of different sizes. The goal of our project was to develop reliable and reproducible procedure for on-site liposome preparation when researcher can make liposomes and use them immediately in their experiments. Using liposomes as reference standards, we propose a technique based on side-scattered light analysis on a flow cytometer to characterize MVs from human serum.
Methods: Liposomes where prepared via new centrifugation technology. They were analyzed by dynamic light scattering (DLS), transmission electron microscopy (TEM) and flow cytometry.
Results: Flow cytometry was used to study fluorescein labeled or blank liposomes of defined dimensions. Their size and structure was confirmed by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Linear dependence of side scattering (SSC) from liposome size was established in the range from 200 nm to 700 nm. However, it was found that liposome light scatter depends on their lipid composition. We use liposomes and polystyrene microparticles for flow cytometry instrument calibration and MV size determination.
Summary/Conclusion: 1. A new technology was utilized to produce a set of liposomes of different sizes ranging from 100 to 700 nm. 2. Dependence of SSC from liposome dimensions has a linear correlation. 3. This set of liposomes can be used for size determination of MVs from human serum.

IP.05

Rapid isolation and miRNA profiling of intact exosomes in colorectal-cancer patients

Jonathan Shaffer[1], Martin Schlumpberger[2], Karolin Spitzer[2], Verena Schramm[2] and Markus Sprenger-Haussels[2]

[1]QIAGEN Sciences; [2]QIAGEN GmbH

Introduction: Fast and reproducible isolation of exosomes and other extracellular vesicles presents a major challenge in exosome research and further hinders downstream analysis. Here, we demonstrate a complete and reproducible workflow from rapid isolation of vesicular-specific RNA, including miRNA and other small RNAs, using a membrane affinity-based procedure in spin column format [1] to efficient profiling and analysis of vesicular miRNA content by next-generation sequencing. This workflow was applied to colorectal cancer (CRC) patients.
[1] Enderle D, Spiel A, Coticchia CM, Berghoff E, Mueller R, Schlumpberger M, et al. (2015) Characterization of RNA from Exosomes and Other Extracellular Vesicles Isolated by a Novel Spin Column-Based Method. PLoS ONE 10(8): e0136133.
Methods: Vesicular RNA from plasma of CRC patients was isolated using a spin column-based approach. Libraries for miRNA-Seq were prepared using a novel ligation-mediated approach for library prep which assigns unique molecular indices (UMIs) to each miRNA. Next-generation sequencing was then performed using a benchtop sequencer. Reads were mapped to miRbase and identical reads were collapsed based on the UMI sequences.
Results: Using the novel workflow, EV-specific miRNA content from serum, plasma and other biofluids can be profiled efficiently with total hands-on time of less than four hours for the complete workflow from isolation of vesicular RNA to miRNA-seq libraries. 35-40% of all reads consistently map to known miRNAs annotated in miRbase. This high percentage of mapped reads results from efficient removal of Y-RNAs and other small RNAs during the library preparation. We find EV-specific miRNAs to be highly abundant among all sequenced miRNAs proving the isolation of EV-specific RNA content.
Summary/Conclusion: We conclude that the combination of a spin-column based EV-specific RNA isolation and miRNA-seq library preparation optimized to remove Y-RNAs is highly suited to accurately profile miRNAs from CRC patients. This approach maximizes the amount of interpretable data to specifically profile miRNAs inside of EVs without background from miRNAs outside of EVs.
Funding: This research was funded by QIAGEN GmbH, Hilden, Germany. CRC patient samples were provided by our collaborator Prof. Dockhorn at Zentrum für Pathologie, Kempten, Germany

IP.06

EXÖBead: A glycan recognition method of isolating exosomes from small sample volumes without ultracentrifugation

Dapi Chiang[1], Dominik Buschmann[2], Benedikt Kirchner[2] and Michael Pfaffl[2]

[1]Biovesicle Inc.; [2]Division of Animal Physiology and Immunology, TUM School of Life Sciences Weihenstephan, Technical University Munich

Introduction: Exosomes are small vesicles (30-150 nm) secreted from different cell types and found in various biofluids, such as blood, urine, saliva and CSF. Exosomes contribute to cell-cell communication, antigen presentation or tumor progression by carrying cellular proteins, RNA/DNA, glycans and lipids. Differential ultracentrifugation (UC) is still regarded the 'Gold Standard' for exosome isolation. However, UC is a laborious and time-consuming method that requires specialized equipment and operational expertise. Several alternative methods such as antibody-conjugated beads were developed to isolate exosomes without UC. Isolation based on antibody-conjugated beads, however may damage exosomes by using acidic or alkaline reagent to break antigen-antibody interactions.
Methods: To solve these issues, we create a "non-antibody" coated bead, called EXÖBead, that is able to isolate exosomes. We incubated EXÖBead with pre-cleared cell culture medium (CCMs) or serum and analyzed the pulled-down fraction by FACS, western blot, Bioanalyzer 2100 capillary electrophoresis, Transmission Electron Microscopy (TEM) and Nanoparticle Tracking Analysis (NTA).
Results: Our result showed that CD63 can be detected by FACS in exosome-bead complexes from 100 µl to 1 ml human serum (MFI: 40.7% to 76.2%). Additionally, the expression of Alix and Rab5 was substantiated by western blot using the exosome-EXÖBead complex from 200 µl mouse serum or 200 µl B16F10 CCMs. The pattern of vesicular RNA and its cDNA was found to be similar for exosomes isolated by EXÖBead and differential UC (120,000 g pellet). For the RT-qPCR study, U6 (28.9 cycles) and miR-33 (34.7 cycles) can be detected in exosomes isolated from 10 ml THP-1 derived macrophage CCMs. Furthermore, we designed an exosome elution buffer without using any acidic or alkaline reagents. To test its ability to release exosomes from beads, we performed NTA and TEM to assess vesicle size and morphology. The size of exosomes from NTA (mode of diameter: 154.3 +/- 4.9 nm) and TEM (diameter: 50 nm to 110 nm) eluted from beads is similar to exosomes isolated by UC.
Summary/Conclusion: In conclusion, EXÖBead can capture exosomes from biofluid samples without ultracentrifugation and exosomes can be successfully eluted from the beads.

IP.07

Activity assays for evaluation of clinical grade MSC-EV anti-inflammatory properties for use in treatment of drug-resistant epilepsy in children

Alessandra Fierabracci[1], Valeria La Marca[1], Kelly Van Wemmel[2], Sally Snyman[2], Silvia Balosso[3], Laura Papetti[4], Maurizio Muraca[5], Annamaria Vezzani[6], Federico Vigevano[7] and Marcin Jurga[2]

[1]Children's Hospital Bambino Gesù, Infectivology and Clinical Trials Area, Type 1 Diabetes Centre; [2]Esperite Cell Factory; [3]Dept of Neuroscience, IRCCS Ist. Ricerche Farmacologiche Mario Negri; [4]Dept of Neurosciences, Children's Hospital Bambino Gesu'; [5]Department of Women's and Children's Health, University of Padua, Padua, Italy; [6]Dept of Neuroscience, IRCCS Ist Ricerche Farmacologiche Mario Negri; [7]Children's Hospital Bambino Gesù, Dept of Neuroscience

Introduction: MSCs exert their biological effects through secretion of extracellular vesicles (EV). We previously showed that MSC-EV have significant immunomodulatory properties. MSC-EV inhibit B cells proliferation/differentiation upon PBMC CpG stimulation, similarly to parent MSCs. Furthermore, MSC-EV induce Treg proliferation/apoptosis and IL-10 secretion, following antiCD3/CD28 PBMC stimulus. In this study we show that clinical grade (CG) EV exert similar

immunomodulation to research grade (RG) counterparts. CG EV could be produced with higher efficiency if compared to RG EV and MSCs manufacturing. Currently our group is preparing MSC-derived EV for clinical tests in treatment of epilepsy, a disorder resistant to anti-epileptic drugs in 40% of children due to neuroinflammation. A novel anti-inflammatory strategy, based on CG EV, is proposed.
Methods: A method of CG EV production is based on human umbilical cord derived (UC) MSCs cultured in a closed, scalable stirred-tank bioreactor system in fully defined GMP culture media. EVs are purified by sequential filtration/sterilization. The final product is analyzed by NTA to evaluate size and quantity, and EVs are characterized by MACSPlex immunophenotyping (FACS), to identify specific CD markers. The immuno-modulatory activity of the CG product is evaluated in comparison with RG EVs and MSCs by specific *in vitro* B and T cells assays.
Results: The CG EV isolation method, has been optimized to obtain at least 1.5 * 10^9 EV/mL in 24 h from 0.1 * 10^6 MSCs. EV diameter cut off is 300 nm. MACSPlex exosome assay revealed that EV are CD9, CD63 and CD81 positive, but HLA-ABC and HLA-DRPQ negative. T and B potency assays, performed on PBMC, indicate immunosuppression by CG EV, similarly to the RG EV obtained from the same MSCs. This effect is revealed by Treg increase, counteracting T eff, upon T cells activation, and by reduction of B cells proliferation and plasma cell differentiation, following B cells activation.
Summary/Conclusion: We have developed and standardized a reproducible method for the production, quantification and immunophenotyping of CG EV, starting from human UC MSCs, with similar immunomodulation if compared to RG EV counterparts. Our data indicate that the use of CG EV could be effective in the treatment of a wide range of immunological diseases, and provide a more accessible alternative for allogenic MSCs.
Funding: Esperite (B)

IP.08

Clinical scale production and wound healing activity of human adipose derived mesenchymal stem cell extracellular vesicles from a hollow fiber bioreactor

John J. Cadwell[1], John Ludlow[2] and Tony Rutt[3]

[1]FiberCell Systems Inc.; [2]Zen Bio; [3]KD Bio

Introduction: Current methods for collecting EVs from conditioned medium use large numbers of tissue culture flasks. EVs are secreted in small quantities and a standard preparation can entail a final stage of 200 T225 flasks or more. This method is wasteful, time consuming, space consuming and the cells are growing in a non-physiologic environment. Hollow fiber bioreactors have been used to produce large quantities of exosomes from human adipose derived MSCs in culture for over two months with no change in phenotype. The harvest volume is 40mls and harvests can be performed 2-3 times a week. Functional assays have shown the bioreactor exosomes were at least as active as the ones obtained from flasks.
Methods: Cryopreserved ASCs (1 x 10^9) were used to seed a 20Kd MWCO PS cartridge (C2011, FiberCell® Systems Inc.) After 48 hours the non-adhered cells were removed from the system. Circulating PM-1 medium was changed when the starting glucose level of 0.45mg/mL was depleted by 50%. A time course of monitoring revealed medium changes need to occur every 3-5 days, which coincided with harvesting the conditioned serum-free medium from the bioreactor cartridge for EV isolation. Cultures were maintained in this manner for three months. *In vivo* biological activity was determined using a model whereby 2cm diameter wounds were made in the back skin of rats and then treated with vehicle control or EVs (25 x10^6 particles). The wounds received a single treatment by topical application onto the surface of the wound and allowing to air-dry for 10 min before covering with a bandage.
Results: A total of 8.60 X 10^{11} EVs were harvested in a total volume of 120 ml over the course of 6 weeks. 130 T225 flasks yielded a total of 1.6 X 10^9 EVs in a volume of 4000 ml. The hollow fiber bioreactor produced the equivalent of nearly 7,000 T225 flasks but in a much smaller volume. The rat wound healing model demonstrated a significant acceleration of the wound healing process.
Summary/Conclusion: Hollow fiber bioreactors represent a more *in vivo* like way to produce exosomes of both the quality and quantity required for clinical relevance in a closed, single use system. MSC phenotype remains unchanged over the course of culture and can produce EVs in a continuous process at a high concentration. EVs produced in this manner demonstrate significant wound healing and biological activity.

IP.09

Urine exosome proteins CXCL9 and CXCL10 are predictors of kidney transplant rejection

Christine M. Coticchia[1], James Hurley[1], Anand Srivastava[2], Esilida Karecci[2], Vasisht Tadigotla[1], Mia Sher[1], Siawosh Eskandari[2], Albana Mihali[2], Jamil Azzi[2] and Johan Skog[1]

[1]Exosome Diagnostics; [2]Transplant Research Center, Brigham and Women's Hospital and Harvard Medical School, MA, USA

Introduction: Approximately 15% of patients with end stage renal disease (ESRD) who undergo kidney transplant will suffer from acute rejection, negatively impacting long-term graft survival and function. Current methods for monitoring rejection, such as serum creatinine and urine protein secretion are not specific and cannot detect subclinical rejection. Kidney biopsy is therefore routinely used to assess acute rejection, increasing both risk to the patient and cost. An accurate, non-invasive method to determine the presence of early, acute kidney rejection would minimize the amount of immunosuppression needed to manage these patients. Exosomal mRNA (exoRNA) and proteins are an ideal source for such biomarker studies. In the transplanted kidney, exosomes originate from glomerular podocytes, renal tubular cells and from immune cells, generated during rejection. Using these exosomes we previously reported the discovery and validation of a 23-gene urinary exoRNA signature for the diagnosis of human kidney transplant rejection. Here we asked if urine exosomal proteins could increase the accuracy, and reduce the number of genes required for the detection of kidney transplant rejection.
Methods: Urine samples were collected from patients undergoing a transplant kidney biopsy for clinical indications. A total of 21 urine samples (10 rejections, 11 non-rejections) were collected from 21 individual patients. Total exosomes were isolated from 10 mL of patient urine and the presence of 92 exosome proteins was determined by Proseek® Multiplex Inflammation, an immunoassay using Olink Proteomics proximity extension assay (PEA).
Results: Among the 92 proteins examined, CXCL9 and CXCL10 were identified to be differentially expressed in both rejection versus non-rejection urine exosome protein and urine exoRNA. Receiver-operating-characteristic (ROC) area under the curve (AUC) analysis determined that urine exosome-associated proteins CXCL9 and CXCL10 could distinguish patients with kidney transplant rejection from those without rejection with an accuracy of 0.827, ($P \leq 0.01$).
Summary/Conclusion: We additionally identified 3 independent exosome proteins that are differentially expressed in patients with and without kidney transplant rejection, demonstrating that urine exosome proteins are a promising source of biomarkers for organ rejection.

IP.10

Many standard urine extracellular vesicle preparations contain significant cellular biomolecule contamination

Anna Markowska, R. Scott Pendergrast, J. Stephen Pendergrast and P. Shannon Pendergrast

Ymir Genomics LLC

Introduction: Urine offers many advantages over blood as a source of the diagnostic and prognostic biomarkers contained in extracellular vesicles (EVs). Its collection is easy and less invasive. It is itself less of a biohazard and does not generate biohazards such as used needles and specimen vials. These advantages suggest the potential for At-Home donation of urine samples for clinical studies via mail. At-home donation would dramatically increase the convenience and thus compliance from patients and decrease costs for clinicians. However, although urine contains far less cells than blood, the number contaminating white blood cells, red blood cells, epithelial cells and podocytes is not negligible and is also highly variable from sample to sample. Delivery of urine samples to a clinical and/or research laboratory via the mail introduces the possibility of cellular rupture and contamination of the extracellular fraction with cellular biomolecules.
Methods: Here we investigate the degree of cellular contamination of EV preparations from "natural" urine samples and from samples spiked with red and white blood cells. We look at both protein and RNA

contamination under a variety of shipping, storage, and experimental conditions. Storage/transport temperatures investigated include Room Temperature, Refrigeration, and Freezing for 0-3 days. Experimental conditions include filtration, cell preservatives, and different low speed spins.
Results: We find that natural samples can contain very significant contamination from proteins and RNA that are highly expressed in blood cells. For instance, the red blood cell miRNA mir-451a can increase >50-fold in samples from women during menstruation. Also, no storage or shipping condition completely protects samples from cellular contamination, including commercial preparations advertised to protect biofluids from cellular degradation. Furthermore, some standard methods for removing cells can actually introduce cellular contamination.
Summary/Conclusion: These findings strongly encourage researchers working with urine samples to take precautions towards preparing truly cell free fractions of vesicles. Possible solutions to this problem will be discussed.
Funding: This study was funded entirely by Ymir Genomics LLC

IP.12

Identification of a one-step scalable method for isolation of extracellular vesicles

Nikki Heath[1], Lois Grant[2], Xabier Osteikoetxea[1], Niek Dekker[1], Lorenz Mayr[2] and Ross Overman[2]

[1]Astrazeneca; [2]AstraZeneca

Introduction: Extracellular vesicles (EVs) have a unique and natural ability to deliver functional cargoes to recipient cells. Exploitation of EVs to deliver therapeutic cargoes such as nucleic acids, small molecules or proteins, to diseased cells is becoming an increasingly interesting and feasible notion. For this to become a reality and enter the clinic, a rapid, scalable and reproducible method of EV isolation will need to be developed. There are some caveats surrounding the current methods for EV isolation. For example the gold standard protocol of differential centrifugation is not readily scalable, and cross flow filtration requires additional subsequent clean-up procedures to isolate EVs in a pure form.
Methods: Here we develop a method by which we use column-based chromatography to isolate EVs in a single step protocol. EVs were isolated by ultracentrifugation, cross flow filtration and ion exchange chromatography from HEK293T cells. Collected EVs were analysed by Western blotting for EV markers, nanoparticle tracking analysis and cryoelectron microscopy.
Results: We have demonstrated that ion exchange chromatography can reproducibly isolate CD63, CD81, ALIX and TSG101 containing EVs from conditioned media. The size distribution of EVs isolated by ion exchange chromatography (mean 179 nm) was similar to that of EVs isolated by ultracentrifugation (mean 160 nm) but not EVs isolated by filtration (mean 123 nm). Although the yield from ion exchange isolation was lower than achieved by filtration (IEX 183 EVs/cell vs. filtration 748 EVs/cell), it was higher than for ultracentrifugation-derived EVs (125 EVs/cell). In addition, unlike cross flow filtration, the isolated EVs did not require further downstream processing to purify the vesicles away from contaminating proteins such as BSA.
Summary/Conclusion: Ion exchange chromatography provides an ideal compromise as an efficient and scalable method for the isolation of clean preparations of EVs in a single step. Further analysis of EVs isolated by ion exchange at a larger scale, together with a better understanding of their *in vivo* characteristics, will be beneficial to determine the extent to which this isolation method could be used within a clinical setting.
Funding: Postdoctoral research scientist AstraZeneca

IP.13

Size Exclusion Chromatography applications: EV isolation from large sample volume

Julia Gavrilova[1], Jekaterina Muhhina[2], Triin Oja[2], Davide Zocco[3], Giorgia Radano[3], Natasha Zarovni[4] and Paolo Guazzi[2]

[1]HansaBioMed Life-Sciences; [2]HansaBioMed Life Sciences; [3]Exosomics Siena; [4]Exosomics Siena SpA

Introduction: Size Exclusion Chromatography (SEC) is emerging as one the most promising methods for isolating and purifying extracellular vesicles (EVs) from different matrices. SEC technique is very efficient for separating EVs from the circulating proteins and does not affect the original shape and functionality of the vesicles, but its use is applicable only to small sample volume (maximum 2 ml, due to the volume capacity of the columns commercially available) limiting negatively the EV recovery from diluted matrices as urine or cell media. HBM-LS has developed a new SEC column for isolating EVs from a large volume of sample and adapted it to different matrices. Additionally, the column separated efficiently the different EV sizes from a single sample.
Methods: EVs isolation was performed from 20 ml of bodily fluids (urine) and cell medium, using ultracentrifugation or SEC. Isolation efficiency, EV size and shape have been assayed with different common techniques (NTA, TEM, ELISA quantification).
Results: SEC had several advantages over ultracentrifugation, including reduced hands-on time and cost, improved ease of use, and higher yield from the same sample volume. Remarkably, the SEC column allowed the separation of EVs of different sizes from the same sample, subsequently characterized by nanotracking analysis and electron microscopy
Summary/Conclusion: The novel SEC column allows EVs isolation from large volume of diluted matrices with higher yield than ultracentrifugation. The protocol enables the separation of EVs of different size suitable for phenotyping or molecular analysis.

IP.14

Nanoparticle tracking (NTA) quantification of fluorescent nanoparticles

Clemens Helmbrecht and Hanno Wachernig

PARTICLE METRIX GmbH

Introduction: Nanoparticle Tracking Analysis (NTA) measures size and concentration in the size range from 10 nm to 1 µm. Physical techniques such as NTA detect particles, however, cannot discriminate whether the detected particles are biological or inorganic particles such as e.g. dust, nano-bubble, metal-oxide particles or precipitates from buffer. To overcome this limitation, NTA is equipped with fluorescence detection capabilities combining the advantages of fluorescence detection and nanoparticle characterization to form fluorescence NTA (F-NTA). Quantification of fluorescent nanoparticles by F-NTA has proven to be challenging, but recently credible results have been obtained as researchers examine what is required to obtain reliable results with exosome samples. Particle Metrix GmbH (PMX) expanded the options available by proper choice of photo-stable dyes as well as instrument design to limit photo bleaching.
Methods: Rapid, reliable and fast acquisition has been performed by short acquisition at several positions by scanning-NTA to avoid photo bleaching of standard fluorophores such as Alexa 488. By scanning through the sample volume, significant statistics can be achieved in a short acquisition time.
Results: Performance of NTA fluorescence detection was verified by means of fluorescent nanoparticle size standards < 40 nm. With quantum nano-dots (Q-dots), lower sizes are achievable. The dynamic range of detection was expanded to the detection of ONE fluorescent PS particle (100 nm) in the presence of 10000 unlabeled particles. Evaluation of methods using membrane dyes such as PKH67, DiO, DiL and CMO are shown on EVs and Liposomes. Quantification of EVs selectively tagged by means of specific antibody labelled with Alexa-Fluor dyes is also shown.
Summary/Conclusion: Although F-NTA was first introduced 6-8 years ago, it has been slow to develop due to challenges tagging with quantum dots, and photo bleaching of standard fluorophores. PMX GmbH has designed an F-NTA instrument that in large part negates the issue of photo bleaching with many fluorophores by quickly scanning through the sample volume with 1-2 second acquisition times.

IP.15

Evaluating limit of detection for fluorescence NTA measurements: experiments with model systems and fluorophores

Agnieszka Siupa, Clayton Deighan, Sonja Capracotta and Duncan Griffiths

Malvern Instruments

Introduction: As interest in extracellular vesicles (EV) continues to grow, the Nanoparticle Tracking Analysis (NTA) technique has proven to be a valuable and effective tool for EV characterization, commonly used for the detection and measurement (size and concentration) of EV's after isolation. By introducing a fluorescence label and using fluorescence mode NTA (fNTA), researchers are able to confirm that the isolated particles are vesicles or identify a particular biomarker to expand upon the current EV characterization methods. To date, fNTA experiments have met with varying degrees of success.
Methods: This paper discusses a critical variable for successful fNTA measurements, the minimum number of fluorophore molecules needed per particle for detection and example experiments to show how to ascertain this value for different fluorophores. Detection of a fluorescently labeled particle is a multifaceted problem related to the intrinsic properties of the dye molecule, the optical arrangement of the instrument, and method of sample preparation. To quantify in specific terms the number of fluorophores required for detection in different systems three model experiment results are presented.
Results: Three separate model systems were evaluated:

- Liposomes (~120 nm) loaded with Atto 550 incorporated at different concentrations.
- Cationic lipoplex nanoparticles (~60 nm) formed with various loadings of Cy3 labeled short RNAs.
- Titration of biotinylated 80 nm gold nanoparticles labeled with streptavidin labelled with NorthernLights™ 557 dye

These model systems provide easily quantifiable approaches to determining number of fluorophores per particle and give results of 160, 35, and 20 fluorophores/particle respectively.

Summary/Conclusion: We discuss these results in the context of exosome labeling experiments, providing the reader with important considerations and experimental design points.
Funding
These experiments were funded as regular work duties of the authors in developing new applications.

IP.16

To the standardization of exosome isolation and characterization

Julia Luciano-Chadee

Beckman Coulter Inc.

Introduction: Research involving exosomes is rapidly expanding with a vast increase in the quality and quantity of publications. An improved and more efficient isolation protocol for exosomes is critical to advancing this exciting filed.
Methods: Challenges to researchers working with exosomes include setting up density gradients by hand, because it is tedious, time consuming and subject to user, lab, and method analysis. At the same time, experts in the field have called for the establishment of standard protocols. This poster focuses on solutions to those challenges through cost-effective, large-scale purification and fast analysis of exosomes.
Results: Specifically, the Beckman Coulter product portfolio helps to overcome human variables and cost while maintaining reliability, reproducibility, and high-throughput.
Summary/Conclusion: Beckman Coulter is a complete exosomes workflow solution that combines methods of centrifugation, automation, flow cytometry, and particle analysis.
Funding: *Beckman Coulter, the stylized logo, and the Beckman Coulter product and service marks mentioned herein are trademarks or registered trademarks of Beckman Coulter, Inc. in the United States and other countries. All other trademarks are the property of their respective owners.*

Room: Harbour Ballroom
Networking Event 8:00 p.m.

Scientific Program ISEV2017
Sunday, May 21, 2017

Experts Workshop Morning Sessions
Room: Metropolitan Ballroom West and Centre
EW- Session-I Workshop on exRNA biology and the analytical methods organised by the ERCC, NIH
Moderator: *Louise Laurent* 7:45–8:45 a.m.

Room: Metropolitan Ballroom East
EW- Session II EV-TRACK
Moderator: *An Hendrix* 7:45–8:45 a.m.

Room: Harbour Ballroom
EW-Session III Demonstration workshop data analysis with FunRich
Moderator: *Suresh Mathivanan* 7:45–8:45 a.m.

Room: Metropolitan Ballroom West and Centre
Session 28 – EVs in Cardiovascular Diseases and Vascular Disorders
Chairs: TBD and Jason Fish

9:00–10:00 a.m.
LBO.24 to LBO.27

LBO.24

Synthetic stem cell microparticles for heart repair

Ke Cheng

UNC-Chapel Hill & NC State University, NC, USA

Introduction: Stem cell therapy faces a number of challenges. It is difficult to expand, store, and transport stem cells before they are administered to the patient. Synthetic analogs for stem cells and acellular approaches are promising to overcome these hurdles and hold the potential to revolutionize regenerative medicine. We sought to fabricate synthetic analogs of stem cells and test their therapeutic potential for treatment of acute myocardial infarction in mice.
Methods: We packaged secreted factors from human stem cells into Poly (lactic-co-glycolic acid) PLGA microparticles and then coated them with stem cell membranes. We named these therapeutic particles "synthetic stem cells".
Results: Synthetic stem cells exhibited a factor release profile and surface antigens similar to those of genuine cells. They promoted cardiomyocyte functions and displayed cryopreservation and lyophilization stability *in vitro* and *in vivo*. In a mouse model of acute myocardial infarction, direct injection of synthetic stem cells promoted angiogenesis and mitigated left ventricle remodeling.
Summary/Conclusion: The synthetic stem cell strategy may provide novel insight into tissue engineering for treating multiple diseases. In my talk I will cover the results recently published (Circ Res 2017; Nature Commun 2017) as well as some unpublished data regarding the vascular delivery and targeting aspect of microparticles and stem cells.
Funding: NIH of USA.

LBO.25

Calpain carried by platelet-derived microparticles cleaves the protease-activated receptor 1 on endothelial cells and initiates vascular inflammation during diabetes

Anastasia Kyselova[1], Ingrid Fleming[1] and Voahanginirina Randriamboavonjy[2]

[1]Institute for Vascular Signaling, Goethe University, Frankfurt, Germany; [2]Institute for Vascular Signaling, Goethe University, Frankfurt, Germany

Introduction: The morbidity and mortality associated with diabetes is related to micro-and macro-vascular complications. The Ca^{2+}-activated proteases or calpains have been implicated in the platelet hyperactivation associated with diabetes. Since calpains are known to be carried by platelet-derived microparticles (PMPs), the aim of the present study was to determine the effect of platelet-derived calpain on the vascular wall.
Methods: Mass spectrometry and ELISA were used to analyse proteins in the culture medium from calpain-treated endothelial cells. Protein levels on the surface of endothelial cells were measured by FACS and en-face immunostaining was used to assess protein expression levels on intact aorta while Western-blot was used to investigate intracellular signaling.
Results: *In vitro* treatment of endothelial cells with PMPs or recombinant calpain 1(CAPN1) led to a decrease in endothelial protein C receptor (EPCR) levels on the cell surface and an increase in its levels in the culture medium. EPCR levels were also increased in plasma from diabetic patients and correlated with plasma calpain activity. Diabetes induction in mice led to increased EPCR levels in the plasma which was prevented by the treatment of the mice with the calpain inhibitor A-705253. Mechanistically, calpain cleaved the protease-activated receptor 1 (PAR-1) on endothelial cells leading to the activation of an intracellular signaling i.e. protein kinase C and extracellular signaling-regulated kinase (ERK) phosphorylation causing on one hand the activation of the Tumor necrosis factor-α converting enzyme and EPCR shedding and on the other hand the increase in the expression levels of Intercellular cell adhesion molecule-1 and leukocytes adhesion as well as cell permeability. All of the calpain effects could be mimicked by PMPs from wild-type but not from $CAPN1^{-/-}$ mice and were abolished in PAR-$1^{-/-}$ endothelial cells.
Summary/Conclusion: These data demonstrate that platelet-derived calpains contribute to diabetes-associated vascular inflammation by targeting the PAR-1 receptor and suggest calpain as a therapeutic target for the prevention of cardiovascular complication of diabetes.
Funding: Deutsche Forschungsgemeinschaft-RA 2435/3-1.

LBO.26

Role of RBC-derived EVs in mediating intercellular communication in murine cardiovascular disease models

Avash Das[1], Olivia Ziegler[2], Shulin Lu[3], John Tigges[3], Vasilis Toxavidis[3], Kirsty Danielson[4], Saumya Das[2] and Ionita C. Ghiran[5]

[1]Massachusetts General Hospital, MA, USA; [2]Mass General Hospital, MA, USA; [3]Beth Israel Deaconess Medical Hospital, MA, USA; [4]University of Otago, Dunedin, New Zealand; [5]Beth Israel Deaconess Medical Center; Harvard Medical Hospital, MA, USA

Introduction: Extracellular vesicles (EVs) function as novel mediators of intercellular communication. Here, we describe a fluorescence switch-based, experimental model to study EV-mediated communication between RBCs and the heart as well as other organs that permits characterization of cross-talk between RBCs and cardiomyocytes at homeostasis and after myocardial infarction.
Methods: Mice with RBC-specific expression of Cre (Erythropoietin Receptor (EpoR) Cre) were crossed with reporter mTmG Rosa26 mice to yield EpoRCre/mTmG off-springs with membrane GFP expression in RBCs and RBC-derived EVs. Cultured dermal fibroblasts from mTmG mice and a mT/floxed/mGFP HEK 293 reporter cell line were used to assess transfer of functional Cre in RBC-derived EVs. To determine targets of RBC-EVs, organs from i) EpoRCre/mTmG (n=3), ii) mTmG (n=3) or iii) mTmG mice transfused with RBC-EVs from EpoR-Cre mice and targets of RBC-EVs (determined by mGFP expression due to Cre-recombination) were assessed by confocal microscopy. Finally, ischemia-reperfusion-infarction (30 min. LAD ligation) was done in EpoRCre/mGmT mice (n=3) and their blood and organs harvested after a span of 4 weeks to analyze changes in quality and quantity of RBC-EV targets following MI.
Results: 1. RBC-EVs (mGFP positive) in plasma accounted for about 9% of total fluorescent EVs as detected by nano-flow cytometry and microscopy. 2. *In vitro* dermal fibroblasts from mTmG mice or mT/floxed/mGFP HEK 293 reporter cells showed mGFP expression with EpoRCre RBC-EVs, suggesting EV-mediated transfer of functional Cre. 3. Cre-mediated recombination was noted in diverse organs in EpoRCre/mTmG mice and mTmTG mice transfused with EpoRCre- EVs with the bone marrow, heart, lungs, kidney and spleen showing the largest

degree of recombined cells. 4. Target profile of RBC-EVs demonstrates a distinct pattern of EV-mediated communication among the organs at baseline that was altered following MI, with increased recombination events in the brain, spleen and peri-infarcted heart.
Summary/Conclusion: We show proof-of-concept for a novel model to study origin and targets of EV-mediated intercellular communication with significant EV-mediated communication between RBCs and cardiomyocytes under homeostatic conditions and following myocardial infarction.
Funding: NIH (NHLBI R01 HL122547 to Saumya Das).

LBO.27

Extracellular vesicles released by induced pluripotent stem cells induce cardiac repair in a model of myocardial infarction/reperfusion

Marta Adamiak[1], Guangming Cheng[2], Sylwia Bobis-Wozowicz[1], Elżbieta Karnas[3], Robert Vincent[2], Michał Sarna[4], Zbigniew Madeja[1], Buddhadeb Dawn[2] and Ewa K. Zuba-Surma[1]

[1]Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; [2]Division of Cardiovascular Diseases, Cardiovascular Research Institute, University of Kansas Medical Center, KS, USA; [3]Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; Malopolska Centre of Biotechnology, Krakow, Poland; [4]Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; Malopolska Centre of Biotechnology, Jagiellonian University, Krakow, Poland

Introduction: Extracellular vesicles (EVs) from different kinds of stem cells have been effectively used to promote cardiac function under pathological conditions. One of the most fascinating characteristics of EVs is their ability to carry cell-type specific mRNAs, miRNAs and proteins. Thus, EVs derived from induced pluripotent stem cells (iPSCs) may provide an interesting alternative to harness the salutary effects of iPSCs and circumvent concerns associated with iPSC research. Here, we aimed to determine the effect of murine iPSC - derived EVs (miPSC-EVs) on the repair of ischemic myocardium *in vivo*.
Methods: EVs were purified by sequential centrifugation of conditioned media collected from the serum- and feeder-free culture of miPSCs. EV morphology, molecular content as well as the influence of EVs on target cell function *in vitro* were carefully characterized. In the *in vivo* study, C57BL/6 mice underwent a 30-minute coronary occlusion followed by reperfusion, and 48 hours later, received intramyocardial injection of vehicle (PBS, n=14), miPSCs (n=15) or miPSC-EVs (n=13). Echocardiography was performed 4 days prior to coronary occlusion/reperfusion and at 48 hours and 30 days after injection. Mice were euthanized at 30 days post injection, heart tissue was harvested and histological analyses were performed.
Results: Cardiac endothelial cells treated with miPSC-EVs exhibited greater angiogenic activity *in vitro* and were more resistant to apoptosis. At day 32 after coronary occlusion/reperfusion, mice injected with iPSC-EVs exhibited significantly improved left ventricular ejection fraction and end-systolic volume compared with vehicle-treated mice ($P < 0.05$). Although iPSC-injected hearts showed improved function and structure, several mice in this group grew teratomas at myocardial injection sites.
Summary/Conclusion: Our data show that intramyocardial injection of miPSC-EVs after myocardial infarction/reperfusion produced similar improvement in cardiac structure and function compared with miPSCs. Thus, we conclude that miPSC-EVs may represent a safer therapeutic alternative to whole cell-based therapy for cardiovascular repair.

Room: Metropolitan Ballroom East
Session 29 - EVs in Immune System and Inflammation
Chairs: TBD and Eric Boilard

9:00–10:00 a.m.
LBO.28 to LBO.31

LBO.28

Exosomes as key regulators of signal relay during chemotaxis

Carole Parent[1], Ritankar Majumdar[2] and Paul Kriebel[1]

[1]Lab Cellular & Molecular Biology, CCR, NCI, NIH; [2]Lab Cellular & Molecular Biology, CCR, NCI. NIH

Introduction: The property of sensing and initiating directional migration in response to external cues or chemotaxis is a fundamental property of biological systems. How cells detect and respond to external chemotactic signals and, in particular, how the spatial and temporal relay of chemotactic signals between cells impact single and group cell migration are key questions in the chemotaxis field.
Methods: Using the social amoebae *Dictyostelium discoideum*, where cAMP acts as a chemoattractant, we have shown that the relay of chemotactic signals between cells is mediated through the release of extracellular vesicles that contain the enzyme responsible for synthesizing cAMP, the adenylyl cyclase ACA. We purified the extracellular vesicles from chemotactic cells and showed that they are exosomal, contain and release cAMP and attract cells in an ACA-dependent fashion. Indeed, mass spectrometry analyses identified many canonical exosomal proteins as well as upstream regulators of ACA. We further show that cAMP is released through specific ABC transporters expressed in exosomes.
Results: We extended our studies to neutrophils and show that LTB4, a key secondary chemoattractant in neutrophils, and its synthesizing enzymes localize to intracellular multi-vesicular bodies that, upon stimulation, release their content as exosomes. Our findings establish that the exosomal pool of LTB4 acts in an autocrine fashion to sensitize neutrophils towards the primary chemoattractant, and in a paracrine fashion to mediate the recruitment of neighboring neutrophils in trans. We also investigated the mechanism by which LTB4 synthesizing enzymes, which are primarily localized on the nuclear envelope, are repackaged in exosomes and provide evidence that lipid homeostasis is involved in LTB4 vesicular packaging.
Summary/Conclusion: We envision that the packaging of chemoattractants in exosomes provides a means of maintaining highly diffusible signals available for long-range cell-cell communication. We foresee that this newly uncovered mechanism is used by other signals to foster communication between cells in harsh extracellular environments.
Funding: This research was supported by the Intramural Research Program of the Center for Cancer Research, NCI, NIH.

LBO.29

Anti-tumor effect of bacterial outer membrane vesicles mediated by interferon-γ

Hyun Taek Park, Kim Oh Youn, Nhung Thi Hong. Dinh, Gyeongyun Go, Lee Changjin and Yong Song Gho

POSTECH

Introduction: Outer membrane vesicles (OMVs) secreted by Gram-negative bacteria is spherical nano-scale membrane vesicles filled with periplasmic contents and currently shed new light on possibility of non-living complex vaccines or delivery vehicles. However, there was no attempt to treat cancer using OMVs. In this study, we investigated bacterial OMVs as a therapeutic agent for treating cancer.
Methods: CT26 (murine colon adenocarcinoma) were grafted subcutaneously to wild type and interferon-γ knockout mice. Following intravenous administration of bacterial OMVs derived from Δ*msbB E. coli* to those mice, tumor volume was measured every 3 days. Tumor tissues obtained are subsequently examined. IVIS analyses are used to monitor targeting of OMVs to tumor tissue. Cytokine levels in blood and tumor tissue lysates are measured by ELISA.
Results: Treatment of *â^†msbB E. coli* OMVs reduced tumor volume in dose-dependent manner and completely eradicated the tumor tissue when 5 μg of *â^†msbB* OMVs are injected ($P < 0.001$, versus no-treated mice; total $n = 14$ mice per group, two independent experiments). Interestingly, IVIS imaging analyses showed that OMVs were highly enriched in tumor tissue rather than spreading out the other organs. Cytokine analyses have revealed that IL-12p40, IFN-γ and CXCL10 cytokines increased in blood and tumor tissue upon OMVs treatment. In addition, OMV treatments of mice with IFN-γ deficiency failed to induce such anti-tumor effect ($P < 0.001$, versus wild type mice; $n = 6$ mice per group).
Summary/Conclusion: We here demonstrated that administration of bacterial extracellular vesicles, especially Gram-negative bacterial OMVs, resulted in a remarkable anti-tumor effect without noticeable side effects. Moreover, we found that anti-tumor effect of OMVs is mediated by interferon-γ dependent manner since tumor in IFN-γ deficient mice were not affected by OMV treatment. Therefore, we here suggest that bacterial OMVs are promise immunotherapeutic agent to treat various cancers and these could bring a new insight in the development of novel immunotherapy in the future.

LBO.30

The particular pathology of systemic lupus erythematosus

Ole Østergaard[1], Julia T. Tanassi[2], Christoffer T. Nielsen[3], Jesper V. Olsen[4] and Niels H. H. Heegaard[5]

[1]Department of Autoimmunology and Biomarkers, Statens Serum Institute + The Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Denmark; [2]Department of Autoimmunology and Biomarkers, Statens Serum Institut; [3]Copenhagen Lupus & Vasculitis Clinic, Centre for Rheumatology and Spine Diseases, Rigshospitale, Copenhagen, Denmark; [4]The Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Denmark; [5]Department of Autoimmunology and Biomarkers, Statens Serum Institute + Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Copenhagen, Denmark

Introduction: The pathogenesis of the autoimmune disease systemic lupus erythematosus (SLE) may be linked to aberrant microparticle (MP) generation and removal leading to inflammatory signaling and subsequent autoimmune reactions and tissue damage. While the removal of normal MPs appears to work efficiently in SLE we previously found increased numbers of Annexin V (AnxV) non-binding MPs in the plasma of SLE patients compared to healthy controls (HC) and other autoimmune diseases. We here characterize the proteomes of MPs and AnxV non-binding MPs from SLE and HC to get insights to the origin and uniqueness of SLE associated MPs (luposomes).
Methods: MPs were isolated from HC (n=4) and SLE (n=4) citrate plasma by repeated centrifugation at 18,890 x g. AnxV non-binding MPs were enriched by negative selection using AnxV-coupled microMACS beads. The total MPs and the AnxV non-binding MPs were then subjected to tryptic digestion and resulting peptides were analyzed by LC-MSMS to identify and quantitate proteins by label free quantitation using

MaxQuant. MP groups were compared after normalization using β-actin levels and results were analysed further in Perseus and Excel.
Results: In the total MPs samples 2,500-3,500 unique proteins were identified while the AnxV non-binding MPs yielded 1,700-3,000 proteins. Hierarchical clustering showed separation between HC and SLE MPs and between HC and SLE AnxV non-binding MPs. Overall, collagen-derived peptides were increased in the AnxV non-binding MPs. Specifically for the SLE AnxV non-binding MP there were increased amounts of complement factors, immunoglobulins, galectin-3 binding protein and other proteins. Conversely, platelet integrins and mitochondrial proteins were increased in total MPs compared to the AnxV non-binding MPs.
Summary/Conclusion: Deep proteome profiling of MPs from SLE patients and HC confirm the presence of a sub-fraction of abnormal MPs in SLE. Furthermore, for the first time we here characterize the extremely different proteome profile of AnxV non-binding MPs from SLE patients that seems to be responsible for the differences in the total MP profiles that are unique for SLE. The characterization of these MPs (luposomes) is of importance for understanding the pathogenesis of SLE, for development of new diagnostic tools, and also provide avenues for the development of new therapeutic concepts.

LBO.31

Presence of diabetes autoantigens in extracellular vesicles derived from human islets

Craig P. Hasilo[1], Sarita Negi[2], Isabelle Allaeys[3], Nathalie Cloutier[3], Alissa K. Rutman[4], Marco Gasparrini[2], Eric Bonneil[5], Pierre Thibault[5], Eric Boilard[6] and Steven Paraskevas[2]

[1]McGill University, Montreal, Canada; [2]McGill University Health Centre, Research Institute of the MUHC, Montreal, Canada; [3]Centre de Recherche du CHU de Québec, CHUL; [4]Research Institute of the McGill University Health Centre, McGill University, Montreal, Canada; [5]Institute for Research, Immunology and Cancer, Université de Montreal, Canada; [6]Department of Microbiology-Infectious Disease and Immunity and Faculty of Medicine, Université Laval, Quebec, Canada

Introduction: The role of extracellular vesicles (EV) in the etiology of type 1 diabetes is poorly understood. We set out to determine if human islets, isolated using clinical transplant protocols, produce EV in culture and if they contain diabetes autoantigens (DAA).
Methods: Islet conditioned media (ICM) was collected from human islets (≥80% purity; cultured up to 72hrs) isolated from non-diabetic donors (n=10) under research consent. Nanoparticle tracking analysis (NTA) and flow cytometry (FC) were used to determine particle concentration and size distribution. ICM (n=10) were labeled with CellTracker Deep Red (CT), annexin V, DAA GAD65, IA-2, and ZnT8, βcell markers Glut2 and Hsc70, EV markers CD9 and CD14 on a FACS Canto II with small particle analyzer. Serial centrifugation at 50 000 g and 200 000 g generated ICM-50K and ICM-200K fractions, respectively. Double-labeled immunogold TEM was performed for AnnV plus GAD65, ZnT8 or Glut2. Proteomic analyses were performed by mass spectrometry on ICM-50K and ICM-200K (n=5 donors), and islet cell lysate (ICL) on a LTQ-Orbitrap Elite. Values obtained in lot-matched unconditioned media were subtracted from experimental values.
Results: Varying levels of EV between donor ICM were detected with a consistent size distribution, and varying antigen levels. Proteomics of ICM-50K and ICM-200K revealed over 56% homology with ICL, but not with an endothelial cell line. Enrichment analyses identified proteins of exosome lineage, associated with diabetes pathways, and capable of eliciting an immune response. GAD65, ZnT8 and Glut2 presence on EV was confirmed with immunogold-EM.
Summary/Conclusion: Morphologically diverse ICM EV, containing varying levels of DAA GAD65, ZnT8, and Glut2, were detected with a consistent size profile between non-diabetic donors. Proteomic evaluation reflected an islet-specific protein signature capable of promoting an immune response. These biomarkers may be strong targets for early diagnostic markers of β-cell injury and interventional strategies in diabetes and islet transplantation.
Funding: Canadian National Transplant Research Program (CNTRP) and the McGill University Health Centre Foundation. C.P.H. is the recipient of the Astellas CNRTP Training Award, and E.B. is the recipient of a new investigator salary award from the Canadian Institutes of Health Research.

Room: Harbour Ballroom
Session 30 - Novel Developments in EV Biogenesis and Characterization
Chairs: An Hendrix and Jeff Franklin

9:00–10:00 a.m.
LBO.32 to LBO.35

LBO.32

Neutral sphingomyelinases control Extracellular Vesicles budding from the plasma membrane

Julia C. Gross[1], Kerstin Menck[2], Can Sönmezer[3], Thomas Worst[4], Matthias Schulz[3], Gry Dihazi[3], Frank Streit[3], Gerrit Erdmann[5], Simon Kling[6], Michael Boutros[7] and Claudia Binder[3]

[1]University Medical Center Gottingen, Germany; [2]INSERM, U1068, Centre de Recherche et Cancérologie de Marseille, France; [3]University Medical Center Gottingen, Germany; [4]Mannheim Medical Center, University of Heidelberg, Germany; [5]NMI TT Pharmaservices; [6]NMI Natural and Medical Sciences Institute at the University of Tubingen, Germany; [7]German Cancer Research Center (DKFZ), Division Signaling and Functional Genomics and Heidelberg University, Heidelberg, Germany, Department of Cell and Molecular Biology, Faculty of Medicine Mannheim, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany; German Cancer Con

Introduction: Extracellular vesicles (EVs) are membrane particles secreted from cells into all body fluids. Several EV populations exist differing in size and cellular origin.
Methods: Using differential centrifugation EVs pelleting at 14,000 g ("microvesicles" (MV)) and 100,000 g ("exosomes") are distinguishable by protein markers. Neutral sphingomyelinase (nSMase) inhibition has been shown to inhibit exosome release from cells and has since been used to study their functional implications.
Results: How nSMases (also known as SMPD2 and SMPD3) affect the secretion of MVs is unknown. Here we investigated how SMPD2/3 impact both EV populations. SMPD2/3 inhibition by GW4869 or RNAi decreases secretion of exosomes, but also increases secretion of MVs from the
plasma membrane. Both populations differ significantly in metabolite composition and Wnt proteins are specifically shifted onto MVs under these conditions.
Summary/Conclusion: Taken together, our data reveal a novel regulatory function of SMPD2/3 in vesicle budding from the plasma membrane and clearly suggests that - despite the different vesicle biogenesis - the routes of vesicular export are adaptable.

LBO.33

Live-cell imaging for neural stem cells-derived exosomes during neurogenesis by exosomal microRNA using a microfluidic device

Hyun Jeong Oh[1], Seok Chung[2], Do Won Hwang[1] and Dong Soo Lee[1]

[1]Department of Nuclear Medicine, Seoul National University, Seoul, Republic of Korea; [2]School of Mechanical Engineering, Korea University, Seoul, Republic of Korea

Introduction: Exosomes are cell-derived vesicles that shuttle miRNAs involved in regulation of cellular process including cell proliferation and differentiation. Neurogenic microRNA (miRNA) such as miR-124 or miR-9 can be transferred and plays essential roles in differentiation of neural stem cells (NSCs) and neural progenitor cells (NPs) to neuronal cells. In this study, we proposed a mode of exosomal miRNA-mediated cell-non-autonomous neurogenesis and visualized the migration of exosomes to neighboring cells using a customized microfluidic assay.
Methods: Real-time imaging of exosomal transport was performed using the GFP-tagged CD63 plasmid vector and GFP-tagged exosomes were monitored by confocal microscopy on microfluidic device. NE-4C, neural stem cells and F11, neural progenitor cells were used to examine exosomes. The pRV-effLuc/3xPT_miR-193a vector was used to detect miR-193a expression which luciferase signal could be turned off by binding of miR-193a to the triplicates of miRNA binding site in the 3' UTR of effLuc.
Results: The miR-193a was highly expressed in differentiated cells and exosomes secreted from those cells after neurogenesis. The miR-193a facilitated neurogenesis in neural progenitor cells and neural stem cells by blocking proliferation-related target genes. Luciferase activity of undifferentiated (UD) recipient F11 cells/effLuc/3×PT_miR-193a was decreased only co-culture with differentiated (D) donor cells after neurogenesis using a microfluidic device. Time-lapse live-cell imaging using microfluidics clearly visualized the convective transport of exosomes from D-donor to UD-recipient cells. Exosomes containing miR-193a from D-donor cells were taken up by UD-recipient cells and lead them to neurogenesis.
Summary/Conclusion: In this study, we established exosome-tracing microfluidic platform to visualize convective exosomal transport from differentiated to undifferentiated cells and validated that exosomes and neurogenic miRNA within these exosomes propagate cell-non-autonomous neurogenesis to neighboring progenitors.

LBO.34

Importance of choroid plexus-mediated extracellular vesicle secretion in the propagation of Alzheimer's disease

Sriram Balusu[1], Charysse Vandendriessche[1], Caroline Van Cauwenberghe[1], Marjana Brkic[1], Bart De Strooper[2], Claude Libert[1] and Roosmarijn Vandenbroucke[1]

[1]VIB-UGent; [2]VIB-KULeuven

Introduction: Increasing evidence indicates that extracellular vesicles (EVs), including exosomes, play an important role in Alzheimer's disease (AD) pathology. We recently reported that the choroid plexus epithelial cells, present at the interface between blood and cerebrospinal fluid (CSF), show increased EV secretion into the CSF upon peripheral inflammation. Moreover, these EVs were able to enter the brain parenchyma thereby spreading a pro-inflammatory message. Here, we studied the importance of choroid plexus-derived EVs in AD pathology.
Methods: We made use of two mouse models of Alzheimer's disease: transgenic APP/PS1 mice and intracerebroventricular (icv) injection of Aβ oligomers (AβO) in wild type mice. EVs were analyzed using NanoSight, electron microscopy and western blot analysis. Several immunostainings with EV markers were performed on brain sections. To assess cognition, we made use of the novel object recognition test.
Results: Analysis of CSF of transgenic APP/PS1 mice revealed that early on in disease progression, there was an increase in amount of EVs compared to age-matched controls. In contrast, no difference in amount of EVs could be observed later on during disease progression. Interestingly, this correlated with an early increase in CSF Aβ. Next, we studied the effect of icv AβO injection and this revealed a significant increase in amount of EVs in CSF. Moreover, we observed that the choroid plexus epithelial cells are an important source of CSF EVs based on *in vitro* analysis of AβO stimulated primary choroid plexus cells and *in vivo* immunostainings and transmission electron microscopy

analysis of choroid plexus tissue. Importantly, we could link the choroid plexus-mediated EV secretion with AβO-induced cognitive decline.
Summary/Conclusion: In conclusion, our results show that AβO induces EV secretion by the choroid plexus and that these EVs play a role in disease spreading and loss of cognition. These data suggest that inhibition of EV production by the choroid plexus might be an interesting therapeutic approach to prevent or treat AD.
Funding: SAO-FRA (Stichting Alzheimer Onderzoek), Research Foundation - Flanders (FWO) and MouseAge COST action.

LBO.35

Rapid isolation of extracellular vesicles using lipid nanoprobes for cancer diagnosis in NSCLC patients

Siyang Zheng[1], Yuan Wan[2], Gong Cheng[2], Xin Liu[3], Si-Jie Hao[2], Merisa Nisic[4], Chuan-Dong Zhu[5], Yi-Qiu Xia[2], Wen-Qing Li[2], Zhi-Gang Wang[2], Wen-Long Zhang[2], Shawn J. Rice[3], Aswathy Sebastian[6], Istvan Albert[7] and Chandra P. Belani[3]

[1]Department of Biomedical Engineering, Micro & Nano Integrated Biosystem (MINIBIO) Laboratory; Dept of Biochemistry and Molecular Biology, University Park, PA; Penn State Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA, USA; [2]Department of Biomedical Engineering, Micro & Nano Integrated Biosystem (MINIBIO) Laboratory, University Park, PA; Penn State Materials Research Institute, University Park, PA, USA; [3]Penn State Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA; Penn State Hershey Cancer Institute, The Pennsylvania State University, Hershey, PA, USA; [4]Department of Biomedical Engineering, Micro & Nano Integrated Biosystem (MINIBIO) Laboratory, University Park, PA; The Huck Institutes of the Life Sciences, University Park, PA, USA; [5]Department of Biomedical Engineering, Micro & Nano Integrated Biosystem (MINIBIO) Laboratory, University Park, PA, USA; The Second Hospital of Nanjing, Affiliated to Medical School of Southeast University, Nanjing, China; [6]Department of Biochemistry and Molecular Biology, University Park, PA, USA; [7]The Huck Institutes of the Life Sciences, University Park, PA; Department of Biochemistry and Molecular Biology, University Park, PA, USA

Introduction: Extracellular vesicles (EVs) released by various cells are lipid bilayer-enclosed entities that can mediate intercellular communication by transferring cargo proteins and nucleic acids. Recently, the pathophysiological roles and clinical values for EVs are under intense investigation. However, most studies are limited by technical challenges in isolating EVs.
Methods: We report a new method for EV isolation that uses a nanoprobe system for spontaneous membrane labeling and magnetic enrichment of EVs in 15 minutes. We use EVs derived from cancer cell line as a model system and compare the isolation performance between the nanoprobe system and ultracentrifugation. Furthermore, we isolate EVs from plasma samples of 19 non-small lung cancer (NSCLC) patients and perform DNA mutation detection.
Results:
A comparison study demonstrates the nanoprobe system offers comparable isolation efficiency, similar cargo composition compared with ultracentrifugation. In addition, it can be readily modified to meet the requirements for various EV and cargo analyses such as EV amount, morphology, cargo contents (DNA, RNA and protein). Further, aided with this system we isolated nEVs in blood plasma from NSCLC patients and successfully identified EGFR and KRAS mutations.
Summary/Conclusion: The efficiency and versatility make this nanoprobe system a new method for EV isolation. Analyses based on this method are conducive to future cancer diagnostics.

Room: Metropolitan West and Centre Featured Abstracts

10:30–11:05 a.m.

SFA-01

Milk-derived extracellular vesicles from non-allergic and allergic mothers differ in T cell modulatory capacity and have a distinct protein composition

Martijn J.C. van Herwijnen[1], Marijke I. Zonneveld[2], Soenita Goerdayal[3], Arianne van Bruggen – de Haan[4], Esther N.M. Nolte-'t-Hoen[1], Johan Garssen[5], Maarten A.F. Altelaar[3], Gerbrich N. van der Meulen[4], Ruurd M. van Elburg[6], Frank A. Redegeld[7] and Marca H.M. Wauben[1]

[1]Department of Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [2]Department of Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands and Division of Pharmacology, Department of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherl; [3]Biomolecular Mass Spectrometry and Proteomics Group, Bijvoet Centre for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences; [4]Department of Paediatric Allergy, Martini Hospital, Groningen, The Netherlands; [5]Division of Pharmacology, Department of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands and Nutricia Research Centre for Specialised Nutrition, Utrecht, The Netherlands; [6]Department of Pediatrics, Emma Children's Hospital/Academic Medical Centre, Amsterdam, Netherlands and Nutricia Research Centre for Specialised Nutrition, Utrecht, The Netherlands; [7]Division of Pharmacology, Department of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands

Introduction: Breast milk is nature's first functional food which contains various bioactive components that modulate the infant's immune system. Early life nutrition is vital for health later in life and breastfeeding may aid in the prevention of allergies. Nevertheless, it has been suggested that the breast milk from allergic mothers can negatively influence the infant's immunity, possibly caused by an altered milk composition. However, due to the complex structure of milk, the molecular mechanism underlying this effect has not been solved. Recently, we and others have identified milk-derived extracellular vesicles (EVs) as an immune modulatory component in milk. In this study, we compared the protein composition and functional T cell modulatory capacity of milk-EV derived from allergic and non-allergic mothers.
Methods: Milk-derived EVs were isolated via differential centrifugation followed by density gradient-based separation of human milk from allergic or non-allergic mothers. Functionality was tested *in vitro* by co-culturing EVs with αCD3/αCD28-stimulated CD4+ T cells. Additionally, LC-MS/MS proteomic analysis was performed to compare the milk-EV proteomes, followed by pathway analysis of proteins that were differentially expressed using MetaCore and ImmuNet.
Results: T cell proliferation, upregulation of activation markers and overall cytokine production were inhibited in the presence of milk-derived EVs, in contrast to T cells that were cultured with milk supernatant depleted of EVs. Remarkably, milk-derived EV from allergic mothers inhibited T cell activation to a lesser extent than EVs from non-allergic mothers. By comparing the proteomes of milk-derived EVs from allergic and non-allergic mothers we found quantitative differences in key proteins between these two groups. These individual proteins linked specifically to the Rac1 and CDC42 signalling pathways, affecting cell proliferation pathways.
Conclusion: These data show that milk-derived EVs differ in their T cell modulatory capacity depending on the allergic status of the mother. The reduced T cell inhibition by EVs from allergic mothers might be due to the relative abundance of key proteins in these EVs.

SFA-02

Characterising extracellular RNA inside and outside of vesicles

Dmitry Ter-Ovanesyan[1], Emma J.K. Kowal[2], Aviv Regev[3] and George M. Church[4]

[1]Harvard University, MA, USA; [2]Massachusetts Institute of Technology, MA, USA; [3]Broad Institute/MIT, MA, USA; [4]Harvard Medical School, MA, USA

Introduction: Exosomes contain a variety of RNAs, including both protein-coding messenger RNAs (mRNAs) and non-coding RNAs. Previous reports have found that for extracellular microRNAs, some exist inside vesicles whereas others are contained outside of vesicles in protein complexes. It is unclear what proportion of extracellular RNA resides inside vs. outside of vesicles.
Methods: We have used differential ultracentrifugation to isolate exosomes from the K562 leukaemia cell line. We then developed a protocol to get rid of RNA not protected by intact lipid membranes by sequential Proteinase and RNAse treatment, resulting in only the RNA inside of the vesicles. We have also verified that this method does not break vesicles. To characterise the resulting RNA inside of vesicles, we have used various techniques such as Bioanalyzer, qRT-PCR and RNA-Seq.
Results: We have found that the majority of RNA (particularly the small RNA fraction) in an exosome pellet isolated by differential ultracentrifugation is not inside vesicles when comparing Bioanalyzer traces of the untreated pellet to the proteinase/RNase treated one. However, our qRT-PCR and RNA-Seq analysis demonstrates that the mRNAs in the exosome pellet are inside the vesicles.
Conclusion: The exosome pellet isolated by differential ultracentrifugation contains RNA that is both inside and outside vesicles. We have developed a protocol to distinguish RNA that is inside of vesicles from that which is outside. We have found that the mRNAs are inside vesicles whereas a considerable portion of the small RNAs are outside of vesicles (presumably in free protein complexes). Identifying RNAs that are truly inside vesicle has important implications for studying the role of exosome cargo in intercellular communication.

LBO.36

Live tracking of endogenous exosome communication *in vivo*

Frederik J. Verweij[1], Philippe Herbomel[2], Graça Raposo[3], Filippo del Bene[4] and Guillaume Van Niel[5]

[1]Exosomes Research Group Department of Pathology VU University Medical Center Cancer Center Amsterdam (CCA), Amsterdam, The Netherlands; [2]Insitut Pasteur; [3]Centre National de la Recherche Scientifique and Institut Curie, PSL Research University, Paris, France; [4]Institut Curie, PSL Research University, CNRS, Paris, France; [5]Institut Curie, PSL Research University, CNRS, UMR 144, Paris, France /Center for Psychiatry and Neuroscience

Introduction: Exosomes are a nano-sized subclass of Extracellular Vesicles (EVs), released by a wide variety of cell types, that have been implicated in many important physiological and pathological processes. Due to the lack of suitable *in vivo* models, however, the *in vivo* dynamics and physiology of exosomes are poorly understood.
Methods: We developed an animal model to study endogenous exosomes *in vivo* by (site-specific) expression of a hCD63-based fluorescent reporter for exosome secretion in zebrafish and used various light- and electron microscopy (LM and EM) techniques for our analysis.
Results: A combination of light- and electron microscopy (LM and EM) techniques allowed us to observe exosome release *in vivo* and track a massive pool of endogenous exosomes in the blood flow of zebrafish embryos. Site specific expression confirmed that these exosomes originated from the Yolk Syncytial Layer (YSL), a multinucleate cell layer in-between the yolk and the developing embryo with essential nutrient transport functions, sharing functional homologies with the mammalian placenta. By Electron Microscopy we observed massive release of EVs from the apical side of the YSL into the blood flow, further confirming

the YSL as major source of (CD63+ve) exosomes in the developing embryo. Next, we used live imaging to track endogenous EVs in the blood flow to identify their main targets. CD63+ EVs where preferentially interacting with endothelial cells in the caudal vein and plexus compared to the caudal artery. EM revealed endocytosis of these EVs in endosomal compartments of endothelial cells. We detected another major fraction of exosomes in the interstitial fluid, suggesting extravasation outside of the vasculature of YSL derived EVs. We finally observed active and specific endocytosis and storage of CD63+ EVs by scavenging macrophages of the caudal plexus.

Summary/Conclusion: Functionally, our data could support a role for YSL derived EVs in nutrient delivery during development, which is our current focus. Altogether, these data reveal for the first time the release, journey and target of endogenous exosomes *in vivo*. We propose the zebrafish embryo as a new model to study endogenous EVs *in vivo* that will open new avenues to unravel fundamental aspects in EV biology.

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Room: Metropolitan West and Centre
Wrap-Up Sessions

11:05–11:35 a.m.

Wrap Up Sessions – Clinical
Speaker: Uta Erdbrugger
Wrap Up Sessions – Basic Science
Speaker: Eric Boilard