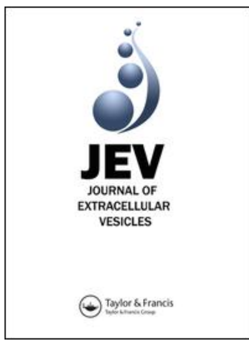

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Journal of Extracellular Vesicles

ISSN: (Print) 2001-3078 (Online) Journal homepage: https://www.tandfonline.com/loi/zjev20

ISEV2019 Abstract Book

To cite this article: (2019) ISEV2019 Abstract Book, Journal of Extracellular Vesicles, 8:sup1, 1593587, DOI: 10.1080/20013078.2019.1593587

To link to this article: https://doi.org/10.1080/20013078.2019.1593587

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JOURNAL OF EXTRACELLULAR VESICLES
2019, VOL. 8, 1593587
https://doi.org/10.1080/20013078.2019.1593587

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The International Society for Extracellular Vesicles is the leading professional society for researchers and scientists involved in the study of microvesicles and exosomes. With nearly 1000 members, ISEV continues to be the leader in advancing the study of extracellular vesicles. Founded in 2012 in Sweden, ISEV has since moved its headquarters to the USA. Through its programmes and services, ISEV provides essential training and research opportunities for those involved in exosome and microvesicle research.

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Advancing extracellular vesicle research globally.

Vision

Our vision is to be the leading advocate and guide of extracellular vesicle research and to advance the understanding of extracellular vesicle biology.

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Plenary Session 1: Standardizations
Chairs: Andrew Hill; Hidetoshi Tahara
Location: Level 3, Hall B

NanoCosmos: extracellular vesicles as nanosized extracellular organelles delivering the complex messages between cells and organisms

Yong Song Gho

Department of Life Sciences, POSTECH, Pohang, Republic of Korea

The secretion of nanosized lipid bilayered extracellular vesicles is a universal cellular process occurring from simple organisms to complex multicellular organisms. Recent progress in this area has revealed that extracellular vesicles play multifaceted pathophysiological functions by delivering the complex messages between cells and organisms, suggesting that extracellular vesicles are NanoCosmos, i.e., extracellular organelles that play diverse roles in intercellular and interkingdom communication. This presentation briefly introduces our last 20 year's comprehensive research on extracellular vesicles derived from host, bacteria, diet and environments including their physical, biochemical and biological complex properties (http://evpedia.info). Then, this presentation focuses on our recent progress in novel extracellular vesicle-mimetic technologies for targeted drug delivery, theranostics and epigenetic reprogramming as well as for adjuvant-free, non-toxic vaccine delivery system against bacterial infection. Furthermore, bacterial extracellular vesicle-based cancer immunotherapy will be introduced. Based on the concept of emergent properties of heterogeneous extracellular vesicles, future research directions to decode the complexity of extracellular vesicle-mediated intercellular communication network, either at the single vesicle level or at a systems level as a whole, and the secret of life will be briefly introduced.

Symposium Session 1: Cardiovascular Disease
Thursday 25 April 2019
Chairs: J. Brian Byrd; Pia Siljander
Location: Level B1, Hall B

11:00–12:30

OT01.01

Extracellular vesicles mediate neutrophil cell deployment from the spleen following acute myocardial infarction

Naveed Akbar and Robin Choudhury

University of Oxford, Oxford, UK

Introduction: Acute myocardial infarction (AMI) mobilizes monocytes from the splenic reserve and induces transcriptional activation *en route* to the injured myocardium, possibly through interactions involving plasma liberated extracellular vesicles (EVs). Neutrophils also reside in the spleen and are the first cells to arrive at sites of injury and mediate further damage. Here, we describe neutrophil deployment from the spleen in AMI and by endothelial cell (EC)-derived EVs.

Methods: Patients provided informed consent as part of the Oxford Acute Myocardial Infarction Study. EV were isolated using ultra centrifugation (120,000*g* 2 h) and characterized for size and concentration by Nanoparticle Tracking Analysis, EV markers (TSG101, ALIX, CD63/CD69) by western blot, and microRNAs (miRNAs) by RT-qPCR. Mouse and human EC were used *in vitro* to derive EC-EV.

Results: Patients presenting with AMI (n = 15) have 2.2-fold more plasma EV at time of injury vs. a 6-month follow-up measurement (P = 0.008). Plasma EVs at the time of presentation correlate significantly with the extent of ischemic injury (R = 0.046, P = 0.006) and plasma neutrophils (R = 0.37, P = 0.017). Experimental AMI in wild type, naïve (C57B6/J) mice induces splenic-neutrophil deployment (P = 0.004). Human plasma EV-miRNAs are significantly altered post-AMI. AMI plasma EV-miRNA-mRNA targets (IPA, Qiagen) are significantly over represented when compared to neutrophil Gene Ontology terms for degranulation (P < 0.001), activation (P < 0.001), chemotaxis (P = 0.008) and migration (P = 0.008). Human EC releases more EV after inflammatory stimulation (control 2.4 × 108 ± 4.9 x 107 EVs/mL vs. tumour necrosis factor-alpha stimulated, 1.4 × 109 ± 3.0 × 108 EVs/mL, P = 0.003) and contains many of the miRNAs enriched in human plasma-EV following AMI. Mouse EC-EV tail vein injected into otherwise wild-type, naïve mice mobilize splenic neutrophils to peripheral blood (P < 0.001).

Summary/Conclusion: Neutrophils appear at sites of injury in the immediate hours after ischemic injury. Neutrophil interactions with EC-EV may mediate their splenic liberation and transcriptional programming following AMI, en route to the injured myocardium. The splenic neutrophil reserve may be a novel therapeutic target in AMI.

Funding: British Heart Foundation.

OT01.02

***In vivo* characterization of endogenous cardiovascular extracellular vesicles and their response to ischaemic injury**

Aaron Scott[a], Costanza Emanueli[b] and Rebecca Richardson[c]

[a]University of Bristol, Uffculme, UK; [b]Imperial College London, London, UK; [c]University of Bristol, Bristol, UK

Introduction: Cardiomyocytes and endothelial cells are counted among the cell types that secrete extracellular vesicles (EVs). EVs mediate the targeted transfer of lipids, proteins and nucleic acids by traversing the extracellular milieu. Recent studies suggest that EVs play a functional role in cardiovascular disease and cardiac repair. For example, a population of exosomes carrying proangiogenic miRNAs was found in the pericardial fluid of patients undergoing heart surgery. Further investigation will be required to determine which cardiac cells are producing these EVs, the cell type receiving them and the functional relevance of this.

Methods: A complete understanding of this process requires a comprehensive *in vivo* model. The zebrafish is an amenable vertebrate model with genetic tractability and optical transparency allowing for subcellular observation in a living organism. The use of stable transgenic lines with cell-type-specific promoters driving the expression of membrane tethered fluorophores allows labelling of the cell membrane and the EVs produced by individual cell types. Light sheet microscopy permits cardiovascular-specific EVs to be tracked *in vivo* and an established ischaemic injury model

allows EV profiles from uninjured, injured and repairing/regenerating cardiac tissue to be determined and compared.

Results: Live imaging of transgenic zebrafish with endothelial cell-derived EVs labelled with mCherry reveals large numbers of EVs in the peripheral circulation, interactions with downstream endothelial cells and release in to the blood flow from filopodia-like protrusions. Cardiomyocyte-derived EVs are observed in the pericardial fluid surrounding the heart and are often seen interacting with cells of the pericardial wall. Additionally, a modified FACS protocol reveals how cardiomyocyte-derived EV numbers fluctuate in response to cardiac injury.

Summary/Conclusion: This data present exciting opportunities to further dissect the cargo being carried by these EVs in a vertebrate model of human disease.

Funding: British Heart Foundation.

OT01.03

Enhanced fibrinolysis and altered extracellular vesicles after remote ischaemic preconditioning in non-diabetic coronary artery disease patients

Caroline J. Reddel[a], Jerrett Lau[b], Gabrielle Penning[c], Vivien Chen[d] and Leonard Kritharides[e]

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Introduction: Brief non-harmful ischaemia, remote ischaemic preconditioning (RIPC) has been shown to confer benefit to patients with coronary artery disease (CAD). Some studies indicate lesser benefit in patients with diabetes. RIPC may enhance fibrinolysis.

Hypothesis: RIPC causes an increase in fibrinolytic potential through release of fibrinolytic factors from the endothelium or fibrinolysis-supporting extracellular vesicles (EVs) and this effect is less evident in patients with diabetes.

Methods: Patients at Concord Hospital with suspected CAD gave written informed consent and were administered RIPC (sphygmomanometer on the arm, 3 × 5 min cycles, n = 31) or sham (n = 29) before angiography, with recruitment ongoing. Blood was collected pre- and immediately post-RIPC/sham and platelet-free plasma generated. Global coagulation/fibrinolytic potential was measured by overall haemostatic potential assay (Reddel et al. Thromb Res. 2013; 131(5): 457–462) and various fibrinolytic factors by ELISA. EV were assessed by flow cytometry (Reddel et al. Thromb Haemost. 2018; 118(4): 723–733) using fluorescent surface markers for phosphatidylserine and cell origin including platelets (CD41a), leukocytes (CD45) and MAC-1 (CD11b). Positive events were defined with supernatant of ultracentrifuged pooled normal plasma as negative control. Changes pre–post RIPC were assessed by paired t-test. The study was approved by the local ethics committee.

Results: In the whole population, there was no effect of RIPC on fibrinolytic factors but a decrease in platelet-derived EV. However, in non-diabetic patients and not in diabetic patients, RIPC increased overall fibrinolytic potential and CD45+ and CD11b+ EV. These effects were not seen after sham treatment.

Summary/Conclusion: There is a global increase in fibrinolytic potential after RIPC treatment in CAD patients without diabetes mellitus, which may be contributed to by increased leukocyte-derived EV and/or decreased platelet-derived EV. Ongoing work aims to directly identify this contribution in patients who undergo RIPC.

OTO1.04

Urinary extracellular vesicle concentration, microRNA-155 expression and inflammatory surface marker expression are altered in patients with symptomatic coronary artery disease

Stephen Fitzsimons[a], Silvia Oggero[b], Niall Mahon[c], Nicola Ryan[c], Mauro Perretti[d] and Orina Belton[a]

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Introduction: Urinary extracellular vesicles (uEVs) (exosomes, microvesicles and apoptotic bodies) have potential as diagnostic and prognostic biomarkers. In atherosclerosis, the underlying cause of heart attack and stroke, EV release can be dysregulated and their contents can mediate pro-inflammatory effects. Several markers have been previously identified on uEV including exosome markers CD63 and CD9, CD45 (leukocyte marker), CD61 (platelet marker), CD14 (monocyte/macrophage marker) and α/β integrins. The selectively packaged cargo of these membrane bound carriers include microRNAs (miRs). miR-21 and miR-155 are key regulatory miRs that are upregulated in immune cells and are released in EVs following exposure to pro-inflammatory stimuli. miR-155 has been reported to have pro-atherogenic effects and miR-155 deficiency in murine models results in reduced atherosclerotic lesion burden.

Methods: Urine was collected from patients diagnosed with coronary artery disease (CAD), classified as symptomatic (non-ST-elevation myocardial infarction, ST-elevation myocardial infarction or unstable angina) or asymptomatic (stable angina). uEVs from symptomatic and asymptomatic patients were isolated via benchtop centrifugation. The concentration and size of uEVs were analysed via the NanoSight NS300 (n = 15 per group). The expression of miR-155 and miR-21 was investigated by RT-qPCR (n = 10 per group). uEV surface marker expression was analysed by ImageStreamX MK2 Imaging Flow Cytometer (12 per group).

Results: uEV concentration in symptomatic patients (median; 6.46E+9 particles/mL) was significantly decreased ($p < 0.05$) compared to asymptomatic patients (median; 1.25E+10 particles/mL). CD11B+ uEVs were increased and CD16+ uEVs were decreased in the symptomatic patients ($p < 0.01$). In addition, the concentration of CD45+ EVs were increased in symptomatic patients ($p < 0.001$). Although uEV miR-21 was unchanged, miR-155 expression was significantly increased in the symptomatic group ($p < 0.05$).

Summary/Conclusion: uEV concentration, miR-155 expression and surface marker expression have diagnostic and prognostic potential. As CAD severity increases, uEV concentration is reduced, surface marker expression is altered and uEV miR-155 expression is increased.

Funding: The Irish Research Council.

OT01.05

Circulating extracellular vesicle-associated microRNAs as predictive biomarkers of cardiovascular complications in end-stage renal disease

Dakota D. Gustafson[a], Jessica Fitzpatrick[b], Jason Fish[c] and Rulan Parekh[b]

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Introduction: Chronic kidney disease affects roughly three million Canadians and is accompanied by considerable human suffering, premature death and costly medical care. The incidence of ESRD and its associated cardiovascular (CV) complications continues to rise despite improved treatments for the primary etiologies of diabetes and hypertension.

Methods: We are using multiple biological approaches to better understand the organ-level effects of extracellular vesicle (EV)/miRNA dysregulation and gather the detailed mechanistic insight necessary for the development of an integrative CV risk prediction model for diabetic ESRD patients. The foundation of this study is the Predictors of Arrhythmic and Cardiovascular Risk in End Stage Renal Disease (PACE) cohort (n = 571); a cohort of ESRD patients followed over four years, with a repository of clinical data with matched biological samples. ExoQuick was used for the isolation of EVs from plasma, and their properties characterized through NanoSight analysis, western blotting and electron microscopy. A high-throughput microfluidics RT-qPCR platform (n = 420) was used to examine potential associations between miRNA and clinical outcomes. We then validated functionality on human coronary endothelial cells (n = 12, coronary artery disease vs. control) using mRNA-Seq.

Results: EV analysis revealed diabetic ESRD patients have increased circulating vesicle concentrations; in addition to having vesicles of a larger mean size. Data from our clinically translatable microfluidics-based RT-qPCR methodology identified numerous potential microRNA biomarkers for CV complications of ESRD. Following miRNA identification, we utilized penalized regression models to generate a panel of miRNAs which may serve as CV-risk predictors for diabetic ESRD patients. In particular, miR-23b-3p appears to be significantly associated with coronary artery disease severity.

Summary/Conclusion: This data will be weighted with the novel biomarker data and fully integrated to build a clinical risk prediction model for the development of CV complications in ESRD and reassessed in a new cohort (D4 Cohort) replicate the findings and validate the risk prediction model.

Funding: This work was funded by AstraZeneca and the Canadian Vascular Network.

OT01.06

Live tracking system for endogenous exosomes

Weijia Luo[a], Yuan Dai[a], Kelsey Andrade[a], Megha Chandna[b], Pamela Ulloa-Franco[c] and Jiang Chang[a]

[a]Texas A&M University College of Medicine, Houston, USA; [b]University of Texas-Austin, Houston, USA; [c]Trinity College, Hartford, USA

Introduction: Exosomes are emerging new category of messengers that communicating among cells, tissues and organs. Understanding the kinetic of exosome communication *in vivo* is a critical foundation for studying exosome functions and developing exosome-based drug-delivery models. Current studies of exosome *in vivo* trafficking largely rely on the administration of exogenous exosomes labelled by fluorescent

dyes or proteins. These methods may not fully represent endogenous exosome kinetics due to *ex vivo* exosome manipulation. Here, we established the first inducible endogenous exosome tracking mouse model that tracks endogenous exosome released by cardiomyocytes *in vivo*.

Methods: We generated a transgenic mouse expressing the bioluminescent reporter Nano-luciferase (NanoLuc)-fusion protein. The ultrasensitive and stable NanoLuc reporter has a 150-fold stronger signal compared to the traditional Firefly and Renilla luciferases and the longest luminescent half-life amongst all known luciferases. We fused NanoLuc reporter with exosome surface marker CD63 for specific labelling of exosomes. Then, the cardiomyocyte-specific αMHC promoter followed by a loxP-STOP-loxP cassette was engineered for precise spatial labelling of exosomes originating from cardiomyocytes. We then crossed the cardiomyocyte-specific mouse with a tamoxifen-inducible Cre mouse (R26CreERT2) to achieve an inducible system. The exosome labelling and distribution were assessed by luciferase assay and noninvasive bioluminescent live imaging.

Results: CD63NanoLuc expression was tightly controlled and only detected in cardiomyocytes upon induction. The endogenous exosomes released from cardiomyocytes were labelled and detected *in vitro* in cell culture supernatant, and *in vivo* in animal plasma. A signature distribution profile of the endogenous cardiomyocyte-releasing exosomes was achieved.

Summary/Conclusion: This exosome tracking model enables elucidating the endogenous exosome trafficking pattern, and allows the study of exosome behaviour under different conditions. It will provide a powerful tool for the exploration of the biological functions, mechanisms and clinical applications of exosomes in a broad spectrum of research.

Funding: AHA Innovative Project Award: 18IPA34180012.

Symposium Session 2: Nucleic Acid Biomarkers in Human Disease
Chairs: Robert Kitchen; Louise Laurent
Location: Level B1, Lecture Room

11:00–12:30

OT02.01

miRNA exosomal biomarkers in brain derived and serum exosomes associated with neurodegenerative diseases

Lesley Cheng[a], Xia Li[b], James Doecke[c], Laura Vella[d], Natasha Vassileff[e], Mitch C. Shambrook[a], Robyn Sharples[f], Saima Zafar[g], Inga Zerr[h], Catriona McLean[i], Malcolm Horne[d], Colin Masters[d], Kevin Barnham[d] and Andrew Hill[j]

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Introduction: Several blood-based tests have been explored to detect Alzheimer's disease (AD) and other neurogenerative diseases; however, evidence is required to determine whether blood sampling is an appropriate specimen to diagnose brain diseases. Exosomes are small extracellular membrane vesicles packaged with RNA and protein cargo. 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), PD (n = 8), ALS (n = 7) and control (n = 5–8 per group) brain tissues using 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 biomarkers, identified in our previous study, were detected in BDEs, together with differentiators for PD, ALS and CJD diagnosis in serum and in some cases neural-derived exosomes.

Summary/Conclusion: This work has identified highly specific panels of miRNA that is both present in the brain and blood of AD, PD, ALS and CJD patients. The miRNA candidates can be used to develop a blood-based diagnostic test highly relevant to a brain disease, equivalent to noninvasive brain biopsy.

Funding: National Health and Medical Research Council, Australia MND, Australia Creutzfeldt-Jakob disease Support Group Network, Australia Dementia Centre for Research Collaboration, Australia

OT02.02

Brain-derived extracellular vesicle microRNA signatures associated with in utero and postnatal oxycodone exposure: Implications for altered synaptogenesis

Victoria Schaal[a], Dalia Moore[b], Peng Xiao[a], Sowmya V. Yelamanchili[b], Gurudutt Pendyala[a]

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Introduction: Oxycodone (oxy) is a semi-synthetic opioid commonly used as a pain medication which also is a widely abused prescription drug. While very limited studies have examined the effect of *in utero* oxy (IUO) exposure on neurodevelopment, a significant gap in knowledge is the effect of IUO compared with postnatal oxy (PNO) exposure on synaptogenesis – a key process in the formation of synapses during brain development in the exposed offspring. In the present study, we isolated and characterized brain-derived extracellular vesicle (BDE)-associated microRNA cargo from the brains of IUO and PNO offspring using RNA seq. Several key miRNAs unique to both the IUO and PNO groups were identified and validated using RT-PCR. To further gain mechanistic insights, we characterized the miRNA cargo effects on changes in synaptic architecture using *in vitro* primary neurons during a key stage of brain development.

Methods: Density gradient EV isolations from brain tissue, transmission electron microscopy, RT-PCR, *in vitro* primary neuronal cultures and spine density analysis.

Results: Transmission electron microscopy revealed an increase in BDE sizes in both the PNO and IUO groups suggesting that oxy exposure can affect BDE size thus indicating differential expression of molecular cargo.

Next, RNA-Seq identified novel and distinct BDE miRNAs unique to IUO and PNO which were further validated by RT-PCR. Bioinformatics analysis on these differentially expressed BDEs, revealed key Gene Ontology terms involved in neurodevelopment such as neuron projection development, neuronal morphogenesis, pallium/cerebellum development in the IUO offspring. To determine, if BDEs impacted the synaptodendritic architecture, we treated 14 days *in vitro* rat cortical primary neurons with equal amounts of P14 BDEs from the three groups. Confocal imaging of dendritic spines showed a significant reduction on treatment with PNO BDEs and which was further exacerbated on treatment with the IUO BDEs.
Summary/Conclusion: We conclude that BDEs from PNO and IUO offspring carry potentially distinct BDE miRNA cargo that subsequently damage the synaptodendritic architecture and could further lead to neuronal dysfunction at a key stage of neurodevelopment.
Funding: Start-up funds and NIH/NIDA.

OT02.03

Development of a high-performance urine exosomal-mRNA signature for identification of bladder cancer

Sudipto Chakrabortty[a], Robert Kitchen[a], James Hurley[a], Georg Stoll[b], Xuan Zhang[c], Mikkel Noerholm[d], Seth Yu[a] and Johan Skog[e]

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Introduction: Blood in the urine is a common symptom of bladder cancer but of individuals who present with haematuria on average only 8% will have cancer. Moreover, up to 70% of patients with a prior bladder tumour will experience a relapse. The majority of these individuals will therefore undergo invasive and expensive testing (cystoscopy & CT scan) to confirm the presence of a tumour, either for first diagnosis or active surveillance of recurrence. A low-cost, noninvasive urine test capable of preventing unnecessary biopsies is a challenging but attractive proposition.
Methods: Here, we present results from a clinical study in which exosomal mRNAs were profiled from voided urine, collected prior to diagnosis, from individuals suspected of having either newly diagnosed or relapsed bladder cancer. We selected 81 individuals for the clinical study, 44 of whom were diagnosed with mostly early stage bladder cancer. The remaining individuals were healthy or diagnosed with a non-cancerous urinary disease.
Results: We identified a 16-mRNA signature by mining over 25,000 public and proprietary RNA-seq datasets, using a machine learning approach to rank genes based on dysregulation in bladder cancer, presence in urine exosomes and stability to haematuria. Using this signature, we trained a classifier to differentiate samples based on presence/absence of bladder cancer, optimized for negative predictive value (NPV). The model performs well in both newly diagnosed and recurrent cases, even in low-grade disease, with an overall performance of 100% NPV at 46% specificity. As the model is based solely on exosomal mRNA abundance, the score provides entirely new information that would enable a clinician to further improve specificity by considering standard of care parameters.
Summary/Conclusion: Exosomal mRNAs have been used to diagnose other malignancies but this represents the first application of this form of liquid biopsy to bladder cancer. While performance must be validated in a larger clinical trial, this signature could prevent ~50% of unnecessary biopsies, provide a noninvasive means of monitoring relapse and reduce the financial burden of early stage bladder cancer care.

OT02.04

Genome-wide methylation profiling of extracellular vesicle DNA allows brain tumour classification

Franz Lennard. Ricklefs[a], Cecile Maire[b], Katharina Kolbe[b], Mareike Holz[b], Manfred Westphal[b], Ullrich Schüller[b] and Katrin Lamszus[b]

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Introduction: Genome-wide methylation profiling has recently been developed into a tool that allows subtype tumour classification in central nervous system (CNS) tumours. Extracellular vesicles (EVs) are released by CNS tumour cells protecting their cargo, including DNA, from degradation rendering EVs as optimal biomarkers to define subgroups, stratify patients and monitor therapy by liquid biopsy. It is unclear, however, if DNA derived from glioma EVs reflects genome-wide methylation profiles and mutational statuses that would allow tumour classification.
Methods: DNA was isolated from glioma cell cultures (GSC) EVs, GSCs and matched tumour samples (n = 3). EVs were isolated through differential ultracentrifugation and classified by nanoparticle tracking analysis (NTA), immunoblotting, imaging flow cytometry (IFCM), multiplex EV assay and electron microscopy. Genome-wide DNA methylation profiling was performed using a 850-k Illumina EPIC array and classified by the DKFZ brain tumour classifier.

Results: GSCs secrete diverse EVs as measures by IFCM and multiplex EV assay that are high for common EV markers (a.e. CD9, CD63 and CD81). The range of EVs was 120–150 nm measured by NTA. Genome-wide methylation profiles of GSC EVs in addition to copy number alterations and mutations matched their parental GSC and original tumour sample, being Glioblastoma, IDH wildtype or mutant, with additional subclass analyses. Specifically, MGMT methylation statuses could be obtained through EV DNA.

Summary/Conclusion: Here we report, that EV DNA reflects the tumour methylation class as well as most copy number variations and mutations present in the parental cells and the original tumour. DNA EV methylation profiles may therefore be used to detect and classify CNS tumours.

Funding: FLR received a scholarship of the German Academic Foundation.

OT02.05

Methamphetamine use disorder alters plasma extracellular vesicle characteristics and microRNA expression

Ursula Sandau[a], John Nolan[b], Xiao Shi[c], Tracy Swanson[c], Marilyn Huckans[d], William Schutzer[d], Kylie Sage[e], Jodi Lapidus[f], Jennifer Loftis[d], Aaron Janowsky[g] and Julie A. Saugstad[a]

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Introduction: Methamphetamine's (MA) rewarding properties and addictive potential are correlated with increased synaptic dopamine availability following alterations in dopamine and vesicular monoamine transporter function. We examined plasma extracellular vesicle (EV) number, size, protein markers and miRNA content in human subjects who are actively using MA.

Methods: Plasma samples from 10 adults with active MA dependence (MA-ACT), and 10 non-dependent controls (CTL) were obtained from the Methamphetamine Abuse Research Center Biorepository at Oregon Health & Science University and the VA Portland Health Care System. We used single Vesicle Flow Cytometry to directly measure plasma EV concentration and size. We used size-exclusion chromatography (iZON Science) to isolate plasma EVs. EV total RNA isolated by mirVana™ PARIS™ RNA Kit (ThermoFisher) was analysed on TaqMan® Array Human MicroRNA A + B Cards Set v3.0 (ThermoFisher). MiRNA expression was compared between MA-ACT and CTL using two-sample *t*-tests for miRNA expressed in at least 50% of samples in at least one of the two groups. Tobacco use was controlled for.

Results: The data show that in MA-ACT (n = 5) vs. CTL (n = 5), four of the five MA-ACT have an increase in total plasma EVs relative to all five CTL. The average EV concentration in MA users (2 × 108 ± 4.5 × 107 EV/μL) is trending to increased levels, relative to CTL (7.5 × 107 ± 0.9 × 107 EV/μL). EV counts relative to size show a range of EVs with a mode of ~110 nm in both MA-ACT and CTL plasma, and equivalent median EV size. Of 226 miRNA in the EVs, there are 30 miRNAs that meet have area under the curve (AUC) >0.65 and median difference >1, and 47 miRNAs with AUC >0.65 and mean difference >1. Twenty-three of these miRNAs overlap and are the current focus of target prediction.

Summary/Conclusion: EV miRNA expression in subjects with MA use disorder was significantly different than in control participants, suggesting that MA may affect EV communication among cells. The differential miRNA expression also implicates a role for EVs in behavioural and physiological effects specific to MA and suggests that there may be changes in expression of miRNAs that are relevant to specific drugs of addiction, as well as to a spectrum of drug-mediated addiction disorders.

OT02.06

Use of extracellular vesicles purified from lymphatic exudative seroma as surrogate markers of melanoma residual disease

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Introduction: Liquid biopsies in melanoma patients have the potential to improve prognosis. Exudative seroma obtained in the drainage implanted post-lymphadenectomy has never been explored as a source of biomarkers. The use of circulating extracellular vesicles (EVs) as surrogate markers of residual disease could be a novel and powerful non-invasive tool.

Methods: Exosomes were purified by ultracentrifugation from exudative seroma obtained after lymphadenectomy from stage III melanoma patients, were analysed by nanosight analysis and electron microscopy, and compared to plasma in a total of 92 samples. We profiled the proteomic profiles of exudative seroma- and plasma-derived exosomes by mass spectrometry. Extracellular vesicle-associated nucleic acids (EV-NAs) and analysed BRAFV600E by an allele-specific PCR in exudative seroma samples as a novel parameter to detect residual disease

Results: We found that exudative seroma is a novel biofluid highly enriched in EVs, higher size and increased DNA cargo in comparison to plasma. Proteomic analysis of seroma-derived exosomes demonstrated that they are enriched in melanoma oncogenic pathways together with immune-related pathways; however, proteomic analysis did not allow identify biomarkers of relapse or progression. Importantly, detection of BRAFV600E mutation in seroma-derived EVs obtained 24–48 hours post-lymphadenectomy identified patients at risk of relapse significantly (Log rank p = .0067) in a cohort of 17 stage III melanoma patients followed up for 700 days.

Summary/conclusion: Our data show for the first time that exudative seroma obtained post-lymphadenectomy is a novel biofluid enriched on EVs and DNA that can be interrogated for melanoma markers and BRAF mutation. Analysis of BRAFV600 mutation identified patients at risk of relapse with high significance. These data support that our approach could be a novel factor to detect residual disease right after surgery in stage III melanoma patients. Our analysis could be a novel approach to aid oncologists to identify high-risk groups of patients post-lymphadenectomy and improve patient outcome.

Funding: MINECO, NIH, Starr Foundation, Ramon y Cajal Programme, AECC, FERO foundation, and MINECO-REDiEX.

Symposium Session 3: EVs in Cancer Metastasis and Angiogenesis
Chairs: Kyoko Hida; Alissa Weaver
Location: Level 3, Hall B

11:00–12:30

OT03.01

Stem cell-derived extracellular vesicles increase cancer stem cell sensitivity to tyrosine kinase inhibitors through Akt/mTOR/PTEN-combined modulation

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Introduction: Cancer Stem Cells (CSCs) are a small cell population able to sustain the maintenance and recurrence of tumours. In consideration of the high drug resistance and tumour initiating capability, targeting CSCs represents an important approach to eradicate tumours. We previously showed a pro-apoptotic effect of extracellular vesicles (EVs) derived from human liver stem cells. In this study, we evaluated whether HLSC-EVs could act in synergy with tyrosine kinase inhibitor drugs (TKIs) on apoptosis of CSCs isolated from renal carcinomas.
Methods: We administered HLSC-EVs and TKIs to renal CSCs, as co-incubation or sequential administration. TKIs were also loaded in EVs. Intracellular phosphoproteins were evaluated in the CSC lysates by the magnetic bead-based immunoassays Bio-Plex Pro cell-signalling assay and confirmed by Western Blot analysis.
Results: We found that HLSC-EVs in combination with Sunitinb or Sorafenib significantly increased renal CSCs apoptosis induced by low TKI dose. At variance, no synergistic effect was observed when bone marrow mesenchymal stem cell-derived EVs were used. CSC apoptosis was also enhanced when TKIs were loaded in HLSC-EVs. In particular, renal CSCs chemosensitivity to TKIs was enhanced when HLSC-EVs were either co-administered with TKIs or added after, but not before. By a mechanistic point of view, Akt/mTOR and Erk and Creb intracellular pathways, known to be pivotal in the induction of tumour growth and survival, appeared modulated as consequence of TKIs/HLSC-EVs co-administration as well as by EV post-administration.
Summary/Conclusion: Our results indicated that HLSC-EVs and TKIs have a synergistic anti-tumour effect on renal CSCs inducing an enhancement of apoptosis by a combined effect on intracellular pathways pivotal in the induction of tumour growth and survival. This effect appear to be due to an EV-dependent enhancement of TKI induced mechanisms and not to epigenetic changes induced by EV leading to increased TKI sensitivity. This study provides a rational for a combined use in tumour treatment.
Funding: This study was supported by Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), project IG2012 and by grant no. 071215 from Unicyte.

OT03.02

Exosomal nidogen 1 drives liver cancer metastasis by inducing secretion of tumour necrosis factor receptor 1 from activated lung fibroblasts

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Introduction: Hepatocellular carcinoma (HCC) is an aggressive tumour with metastasis as a signature in the advanced stage. Acquisition of migratory and invasive behaviours is fundamental to cancer cells to metastasize. A supportive microenvironment for the colonization of incoming disseminated cancer cells during metastasis is also indispensable. Exosome shedding has emerged as an important channel for intercellular communication in tumour microenvironment during metastasis.
Methods: Exosomes derived from HCC cell lines were functionally characterized by *in vitro* and *in vivo* assays. Proteomic profiling and expression level of exosomal proteins were analysed by mass spectrometry and enzyme-linked immunosorbent assay (ELISA), respectively. The study of interplay between exosomes, HCC cells and lung fibroblasts were carried out using functional assays, immunofluorescent staining and ELISA.
Results: Exosomes derived from metastatic HCC cells augmented cell migration and invasiveness. In animal model, metastatic-exosomes promoted liver tumour formation, increased incidence of distant metastasis to

lungs as well as facilitated colonization of hepatoma cells in lungs and enhanced the permeability of pulmonary vasculature. Proteomic profiling of exosomes identified nidogen 1 (NID1) as a functional component responsible for the promoting effect of metastatic-exosomes. Our data showed that suppression of exosomal NID1 (exo-NID1) significantly diminished the biological activities of metastatic-exosomes. Apart from HCC cells, exo-NID1 enhanced the growth and induced activation of lung fibroblasts. Tumour necrosis factor receptor 1 (TNFR1), found to be released by lung fibroblast pretreated with metastatic-exosomes, showed potent effect in promoting HCC cell motility. Notably, the level of exo-NID1 was well correlated with the metastatic potential of parental HCC cells. Encouragingly, the level of NID1 in circulating exosomes of HCC late stage patients was higher than those at early stage.

Summary/Conclusion: Our study reveals the novel role of NID1, in the form of exosomes, in HCC metastasis and illuminates the expression profile of exosomal NID1 with clinical significance. Our study implicates that targeting signalling pathway mediated by exosomes of metastatic HCC as a therapeutic strategy for HCC.

Funding: RGC GRF (17,113,116) and SKLLR IRF 2017.

OT03.03

Cancer extracellular vesicles create functional heterogeneity of cancer-associated fibroblasts in gastric cancer

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Introduction: Cancer-associated fibroblasts (CAFs) are the major stromal components in the various types of malignancies. It has been recognized that the functional heterogeneity of CAFs provide an appropriate microenvironment for tumour progression. However, it is still largely unknown how functional heterogeneity of CAF is governed by tumour cells. In this study, we investigated the role of extracellular vesicles (EVs) on the formation of CAF functional heterogeneity.

Methods: We treated EVs derived from high-metastatic diffuse-type gastric cancer (DGC) cells or low-metastatic DGC cells to the fibroblasts. By comparing transcriptome profiles of fibroblasts with the EVs, we sought to understand how high-metastatic DGC cells created an appropriate microenvironment for the metastasis.

Results: Our whole-transcriptome analysis of fibroblasts revealed that high metastatic DGC cell-derived EVs strongly induced the expression of inflammatory chemokines such as CXCL1 and CXCL8. However, it is not observed in the fibroblast treated with EVs from low-metastatic DGC. Interestingly, both cancer-derived EVs did not affect the expression of alpha-smooth muscle actin (α-SMA), a typical marker of myofibroblast phenotype. When fibroblasts were treated with TGFβ, α-SMA was clearly induced but suppressed inflammatory chemokine expression in the fibroblasts. Immunocytochemical analysis of CXCL8 and α-SMA showed distinct populations of activated fibroblasts in the co-culture with high-metastatic DGC, suggesting that functional heterogeneity was generated by EVs. We also identified that various miRNAs including miR-193b were enriched in high metastatic DGC cell-derived EVs to induce chemokine expression in fibroblasts.

Summary/Conclusion: Our findings suggest that the intercellular crosstalk of high-metastatic DGC and fibroblasts via EVs contributes to forming the appropriate tumour microenvironment toward the metastasis.

OT03.04

Extracellular vesicles from obese human adipose tissue alter the invasive and proliferative properties of prostate cancer cells

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Introduction: Obesity increases the risk and aggressiveness of multiple cancers including prostate cancer. Adipose tissue (AT) is a rich source of extracellular vesicles (EVs) that were shown to contribute to vascular and metabolic pathologies. Here we characterized the miRNA and proteome of EV isolated from human visceral (V) and subcutaneous (S) fat of bariatric subjects and explored their mechanistic effects on molecular and functional phenotypes of metastatic prostate cancer cells.

Methods: Paired S and V AT collected intraoperatively were used to isolate EVs by ultracentrifugation (n = 27). DIO-labelled EV-S or EV-V was incubated overnight with PC3-ML metastatic prostate cancer cells. EV uptake, proliferation, migration and invasion were quantified by fluorescence microscopy, BrdU incorporation, wound healing and invasion assays,

respectively. The miRNA and proteome cargo of EVs were measured using the Nanostring platform and LC/MS/MS. Changes in gene expression in recipient PC3-ML cells were determined using Nanostring.
Results: EV-S and EV-V produced similar effects on recipient PC3-ML cells. EVs increased cell proliferation by ~1.8-fold ($p < 0.05$); had no effect on cell migration but dramatically decreased cell invasion by 2.5-fold ($p < 0.01$) compared to untreated controls. Gene expression in recipient PC3-ML cells showed significant two to three fold decrease in expression of 8 MMPs without changes in TIMP expression. Mesenchymal markers Snail and Zeb were also significantly decreased and seven glycolytic and PPP enzymes were 1.5- to 2.5-fold increased. Consistent with these changes, the miRNA cargo of EVs was shown to target all the above pathways and the top pathways detected in the EV proteome were metabolism and energy production.
Summary/Conclusion: AT EVs appear to induce a mesenchymal to epithelial transition in prostate cancer cells. This study reveals a novel role of EVs from human AT on metastasis and suggests a new mechanistic link between obesity and prostate cancer.
Funding: Commonwealth of Virginia Health Research Board.

OT03.05

Novel vesicular mediators of peritoneal metastases

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Introduction: Malignant progression results from a dynamic crosstalk between stromal and cancer cells. Recent data suggest that this crosstalk is mediated by exosomes, nanovesicles secreted by various cell types which allow the transfer of proteins, and nucleic acids between cells. We investigated the potential role of omental fat exosomes in gastric cancer peritoneal metastasis.
Methods: Omental fat exosomes were produced from fresh human omental fat specimens. Proliferation, migration, invasion and chemoresistance were used to evaluate the phenotypic behaviour of omental-exosomes treated gastric cancer cells. Using a comprehensive cytokine array, we identified the proteome of omental-exosomes. Exosomal miRNAs were profiled using NanoString technology. A xenograft model was used to evaluate *in vivo* effects of omental-exosomes on gastric cancer tumour growth.
Results: Initially, we demonstrate a robust uptake of omental fat exosomes by gastric cancer cells. We show that these exosomes enhance gastric cancer cell proliferation, migration and invasion. We also revealed that the number of exosomes is directly related to their effect on gastric cancer cells. We further show that omental fat exosomes induce gastric cancer cellular chemoresistance to platinum-based therapy, and that omental exosomes augment gastric cancer xenograft tumour growth *in-vivo*. Using a cytokine array, we characterized the proteome of omental fat exosomes compared to SC exosomes. miRNA profiling identified several established oncomiRs. These vesicles carry numerous proteins and miRNAs implicated in cellular adhesion and chemotaxis, tumour growth and motility as well as chemoresistance; some of these molecules have been reported as pro-tumourigenic factors in gastric cancer. Finally, we demonstrate that omental fat-exosomes increase the expression of transcription factors, mRNA of extracellular matrix proteins and adhesion molecules within gastric cancer cells.
Summary/Conclusion: These observations demonstrate for the first time the uptake of omental fat exosomes by cancer cells; these vesicles carry different molecules which promote gastric cancer cellular aggressiveness *in vitro* and *in vivo*. Taken together, our data imply that omental fat exosomes might play a role in gastric cancer peritoneal spread.

OT03.06

Non-SUMOylated Cx43 changes the recruitment of cellular components into exosomes switching the role of these vesicles in metastatic melanoma

Adrián Varela-Vázquea[a], María D. Mayán Santos[b], Amanda Guitián-Caamaño[c], Alejandro Castro-Iglesias[d], Tamara Camino Martínez[e], Susana B. Bravo-López[f], María Pardo[g], Teresa Calleja-Chuclá[h] and Eduardo Fonseca[c]

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Introduction: Connexin43 (Cx43), a transmembrane protein involved in cell communication and signalling, has been described as a tumour suppressor factor in melanoma, however its role in disease progression remains under debate. Extracellular vesicles (EVs) released by melanoma cells provide signals and "educate" distant cells. The presence of Cx43 in EVs provides these particles with an additional capacity to exchange small molecules such as RNAs, metabolites or ions with target cells via gap junction channels (GJs).In this study, we have investigated the role of exosomal Cx43 in metastatic melanoma.

Methods: Protein levels and activity were studied by western-blot, immunofluorescence, colony formation and proliferation and migration assays. GJIC by Scrape loading. EVs were isolated by ultracentrifugation and analysed using the NanoSight and electron microscopy. Their content was analysed by mass spectrometry (MS) and by RNA-seq.

Results: Low levels and SUMOylated Cx43 in BRAF-mutant human melanoma cells was associated with cytoplasmic distribution and low incidence of dye coupling (GJIC). Ectopic Cx43 gene expression using vectors restored Cx43 membrane localization, raised GJIC and increased Cx43 in the EVs. EVs isolated from BRAF-mutant melanoma cells overexpressing Cx43 only contains the non-SUMOylated Cx43. When different melanoma cell lines were exposed to exosomes containing Cx43, these EVs significantly decreased cell proliferation and blocked colonies growth. The effect of exosomal Cx43 was compared to the overexpression of the protein. The presence of Cx43 in EVs significantly increased the sensitivity of BRAF-mutant metastatic melanoma to drugs such as BRAF/MEK inhibitors. The RNA and proteomic component identified by RNA-Seq and MS revealed that exosomal Cx43 through its scaffolding function could be involved in the recruitment of proteins and small RNAs to the EVs switching the messages and therefore the role of these EVs in melanoma.

Summary/Conclusion: Our results indicate that exosomal particles containing Cx43 are potent vehicles to combat metastatic melanoma. Further understanding of the role of Cx43 in EVs will have implications for the development of new therapeutic strategies. For instance, we demonstrated their ability as drug carriers to combat metastasic melanoma when these vesicles contain Cx43.

Symposium Session 4: EV Biogenesis I
Chairs: Nobuyoshi Kosaka; Clotilde Théry
Location: Level B1, Hall A

11:00–12:30

OT04.01

Linking the trafficking of CD63 and CD9 to their secretion mechanisms into extracellular vesicles

Mathilde Mathieu[a], José Ignacio Valenzuela[b], Mathieu Maurin[a], Mabel Jouve[a], Nathalie Nevo[a], Gaëlle Boncompain[a], Franck Perez[b] and Clotilde Thery[c]

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Introduction: A major challenge in the study of extracellular vesicles is to characterize and separate the different extracellular vesicle (EV) subtypes of a different origin. Indeed, small EVs from the plasma membrane or from endosomes cannot be separated with the classical EV isolation methods. Moreover, even if some of their molecular mechanisms of secretion are known, it is challenging to find specific mechanisms for one particular subtype (see perspective article: Mathieu et al. Nat cell Biol 2019, in press). Understanding how markers of subtypes of EVs are directed to similar or different EVs could help to differentiate them, eventually to describe their specific functions. At least two different populations of small EVs were previously described, one carrying the three tetraspanins CD63, CD9 and CD81, and one with CD9 only (Kowal et al. PNAS 2016).

Methods: We chose to study in HeLa cells the trafficking of CD63 and CD9 and its link with their secretion in EVs, using the RUSH system to synchronize and follow their post-Golgi trafficking (Boncompain et al. Nat Methods 2012). We used the RUSH system to perform live-cell imaging, electron microscopy, immunofluorescence and flow cytometry analyses at different steps of trafficking, and to analyse EVs secreted after a specific time of trafficking.

Results: Despite their presence in the same EVs, CD63 and CD9 do not traffic to the same final compartments. While CD63 is endosomal, CD9 is located on the plasma membrane. We showed that CD9 could be found transiently with CD63 in intracellular compartments before reaching the plasma membrane (PM), while CD63 goes to the PM before being internalized. By forcing stable expression of CD63 at the PM, or impairing post-Golgi and endosomal trafficking, we observed increased secretion of CD63+ but not CD9+ EVs.

Summary/Conclusion: Our results demonstrate that small EVs can form both at the PM and inside multivesicular endosomes. Our tools can be used to determine the respective effects of drugs and gene silencing on secretion of each of these EVs

OT04.02

Interdependency of the multiple endosomal sorting mechanisms influencing exosome biogenesis

Roberta Palmulli[a], Guillaume van Niel[b], Frederik Verweij[b], Xavier Heilingenstein[a], Eric Rubinstein[c] and Graça Raposo[a]

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Introduction: Exosomes are generated as intraluminal vesicles (ILVs) in the multivesicular endosome (MVE). In the endosomal system, protein cargoes either are sequestered to ILVs by inward budding or exit the system by outward budding. Sorting to ILVs is mediated by various machineries, whose interdependency is poorly understood, and is likely counterbalanced by recycling mechanisms that retrieve protein from MVEs. We have taken profit of the particular role of CD63 in the balance between ESCRT-dependent and -independent biogenesis of ILVs and in the sorting of ApoE in melanoma cells to elucidate the interdependency of different sorting mechanisms influencing exosome composition.

Methods: After siRNA depletion of reported key actors of exosome production, EVs released by melanoma cells were isolated by differential ultracentrifugation and floatation on density gradient and characterized using biochemistry and electron microscopy. ILV biogenesis and sorting of specific cargoes throughout the endosomal system was assessed by immunofluorescence or electron microscopy after high-pressure freezing.

Results: Our data show that melanoma cells secrete subpopulations of exosomes with different density and composition. Investigation of known key regulators of in- or outward budding in MVEs differently affected exosome subpopulations. In particular, CD63

modulates ApoE secretion on exosomes and its cellular localization, suggesting that CD63 is a master regulator of cargo trafficking in the endosomal system.
Summary/Conclusion: Our data highlight that exosomes biogenesis is not only dependent on ILV budding but also on a global regulation of endosomal homeostasis. Our study provides a better perception of the interconnections existing between sorting of cargoes to ILVs and their retrieval from the endosomal system. This broader view is crucial to understand the precise roles of reported regulators of exosomes biogenesis that are broadly used by the community.

OT04.03

A bright, versatile live cell reporter of exosome secretion and uptake

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Introduction: Small extracellular vesicles (EVs) called exosomes affect a variety of autocrine and paracrine cellular phenotypes. Understanding the function of exosomes in these processes requires a variety of tools. We previously constructed a live-cell reporter, pHLuorin-CD63 that allowed dynamic monitoring of exosome secretion in migrating and spreading cells. However, there were some caveats to its use, including relatively low fluorescent expression in cells and the inability to make cell lines that stably express the protein.
Methods: By incorporating a stabilizing mutation in the pHLuorin moiety, M153R, pHLuorin-CD63 now exhibits higher and stable expression in cells and superior monitoring of exosome secretion. Cancer cells stably expressing pHLuorin_M153R-CD63 were imaged using a variety of microscopy techniques including a confocal and wide-field microscopy and a correlative light-electron microscopy.
Results: pHLuorin_M153R-CD63 was exclusively detected in exosome-enriched small EV preparations. Live-cell imaging revealed pHLuorin_M153R-CD63-positive puncta left behind migrating cells suggesting the deposition consists of exosomes. Those puncta and trails were not only positive for other exosome markers such as Alix and TSG101 but also correspond to small EVs observed by a scanning electron microscope. In addition, follower cells exhibited pathfinding behaviour over pHLuorin_M153R-CD63 deposits. Incorporation of mScarlet, a non-pH-sensitive red fluorescent tag, to pHLuorin_M153R-CD63 further improves the ability to track trafficking and secretion of multivesicular bodies (MVBs) in cells allowing visualization of trafficking to the leading edge of migrating cells and uptake of external exosome deposits.
Summary/Conclusion: Using pHLuorin_M153R-CD63 construct, we demonstrate superior visualization of exosome secretion in multiple contexts and identify a role for exosomes in promoting leader-follower behaviour in collective migration. By incorporating a further non-pH-sensitive red fluorescent tag, this reporter allows visualization of the entire exosome lifecycle, including MVB trafficking, exosome secretion, exosome uptake and endosome acidification. This new reporter will be a useful tool for understanding both autocrine and paracrine roles of exosomes.

OT04.04

An explanation for “PS-negative” extracellular vesicles: endogenous annexin-a5 from the cytosol cover externalized phosphatidylserines on plasma membranes

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INSERM ‘ParCC’ Paris-Cariovascular Research Center, Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, and Université Sorbonne, Paris, France

Introduction: Stressed cells shed extracellular vesicles (EVs) thought to bear externalized phosphatidylserine (PS) at their surface and promote inflammation, coagulation and tissue injury. Conversely, endogenous cytosolic annexins, such as annexin-A5, orchestrate vesicle trafficking and membrane repair within multiple cell types, via Ca2+-dependent binding to intracellular PS. We hypothesized that endogenous annexin-A5 binds to PS during vesiculation and gets externalized with PS at the surface of EVs.
Methods: We purified healthy plasma and red blood cells and induced Ca2+-mediated vesiculation. We assessed annexin-A5 and EV distribution in supernatants by Western blots, FACS, ELISA, cryo-TEM.
Results: (1) About 20% cytosolic annexin-A5 leaked out during vesiculation, but cytoskeletal proteins were not released. (2) We separated supernatant EVs from “free” proteins by size-exclusion chromatography and quantified EV-bound vs. “free” annexin-A5. All annexin-A5 remained bound to EVs. Other cytosolic proteins (haemoglobin) bound to EVs only partly. FACS with anti-annexin-A5 antibodies revealed the presence of annexin-A5 at the EV surface. (3) We measured EV-bound and “free” annexin-A5 in plasma, vs PS-, PS+, CD235a+ and annexin-A5+ EVs, and made similar observations. Our study suggests that endogenous annexin-A5 can cover externalized PSs on EVs in the presence of Ca2+.

Summary/Conclusion: This new mechanism of PS-neutralization may explain previous reports of apparently "PS-negative" EVs. Conventional detection of EVs with EXOgenous fluorescent annexin-A5 (FACS) may thus depend on PS not being engaged by endogenous annexin-A5 prior to detection. The physio-pathological relevance of endogenous PS neutralization may complement enzyme- and ATP-mediated internalization of PS in healthy cells. PS neutralization may become critical when internalization mechanisms are overwhelmed, and serve to restrain PS-mediated reactions and enforce anti-inflammatory and anti-thrombotic control when the integrity of a few cells only is compromised. On the other hand, dysfunctional annexin-A5 or calcium metabolism may contribute to the release of pro-inflammatory and pro-thrombotic PS+ EVs.

Funding: Funded by Fondation pour la Recherche Médicale and Sorbonne University.

OT04.05

Identification of EV secretion-associated gene involved in melanoma progression by microRNA-based screening

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Introduction: It has been shown that extracellular vesicles (EVs) derived from cancer cells dictate their surrounding microenvironmental cells or distant cells in the future metastatic organs for the benefit of cancer cells. Thus, revealing the molecular mechanisms underlying the production of EVs would prove to be a valuable contribution for establishing EV-targeted therapy against cancer. However, the precise mechanism of EV production, especially in cancer cells, remains unclear. Here, we established a microRNA-based screening system to identify the molecules involved in EV production from melanoma cells.

Methods: Melanoma cell lines, A375 cells, were used in this study. Combined with the ultra-sensitive EV detection method (Yoshioka), ExoScreen, we have screened nearly 2000 miRNAs in melanoma cells. To confirm the results of ExoScreen, we employed the nanoparticle tracking analysis. Target genes of miRNAs were identified by the combination of gene expression analysis and target prediction bioinformatics.

Results: miRNAs which suppressed the secretion of EVs from melanoma cells were identified after the screening of nearly 2000 miRNAs. To understand the molecular mechanisms mediated by these miRNAs, the target genes of these miRNAs were identified and evaluated for their contribution to EV production in cancer cells. Indeed, attenuation of these target genes declined the secretion of EVs from melanoma cells, suggesting the contribution of these genes in EV production/secretion. Furthermore, the expression of these genes was higher in melanoma tumour tissues compared with that in normal tissues.

Summary/Conclusion: These findings suggest that the miRNAs and their target genes were involved in EV production/secretion, resulting in the promotion of cancer progression.

OT04.06

Distinct mechanisms of microRNA sorting into cancer cell-derived extracellular vesicle subtypes

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Introduction: Extracellular vesicles (EVs) encompass a variety of vesicles secreted to the extracellular space. EVs have been implicated in promoting tumour metastasis but the molecular compositions of tumour-derived EV sub-types and the mechanisms by which molecules are sorted into EVs remain mostly unknown. As such dissecting different EV sub-populations and analysing the molecular mechanisms behind active cargo sorting is needed.

Methods: The highly metastatic breast cancer cell line, MDA-MB-231, was used as the model cell line for this study. Iodixanol linear gradient allowed for the separation of EV sub-populations. miRNA profiling and TGIRT-sequencing was used to study the miRNA content of the distinct EV sub-populations. Cell fractionation and cell-free miRNA packaging reconstitutions, coupled with *in vivo* confirmation, in cultured cells, were used to study the molecular mechanisms of miRNA sorting.

Results: We found that at least two distinct EV sub-populations are released by MDA-MB-231 cells. Their differential biochemical properties suggest different sub-cellular origins (endosomes vs. direct budding from the plasma membrane). Moreover, they are governed by distinct mechanisms of miRNA sorting (active vs. passive). By using biochemical and genetic tools, we found that the Lupus La protein is responsible for mir122 sorting into EVs *in vitro* and *in vivo*. Moreover, *in vitro* studies

showed that the Lupus La protein interacts with mir122 with very high affinity. Finally, we uncovered the mir122 motifs required for mir122-La high affinity interaction, and therefore mir122 sorting into EVs.

Summary/Conclusion: Two EV sub-populations with distinct sub-cellular origins, are released by MDA-MB-231 cells. Their differential sub-cellular origin is coupled with two distinct mechanisms of miRNA sorting. The Lupus La protein is responsible for the active sorting of mir122 into EVs *in vitro* and *in vivo*.

Funding: Howard Hughes Medical Institute (HHMI).

Oral with Poster Session 1
Chairs: Uta Erdbrügger; Kenneth Witwer
Location: Level B1, Hall B

13:30–15:00

OWP1.01=PS10.10

miR-1227 alters extracellular vesicle shedding

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Introduction: Extracellular vesicles (EVs) play a key role in cancer development and metastasis by influencing the behaviour of the primary tumour and by aiding the establishment of a pre-metastatic niche in distant organs. This process is due to the EV-mediated functional transfer of biologically active molecules including microRNA (miRNA). miR-1227 is a poorly characterized miRNA that is enriched in EV secreted by prostate cancer (PC) cells in comparison to non-tumorigenic prostate epithelial cells. However, the role of miR-1227 in cancer is poorly understood. Our objective is to determine the role of miR-1227 in PC.
Methods: RNA sequencing from miR-1227 stably expressing PC cells, RISCTRAP Immunoprecipitation of miR-1227 bound mRNA and five different in silico miRNA target prediction methods were used to identify putative miR-1227 targets. Exosomes and large oncosomes (LO) were isolated by differential ultracentrifugation followed by density gradient purification. Atomic force microscopy and TRPS were used to quantify exosomes and LO secreted by PC cells stably expressing miR-1227 or vector control.
Results: A comparative analysis between different EV subtypes indicates that miR-1227 is enriched in LO, a class of EV that are secreted by highly invasive and metastatic amoeboid-migrating cells. LO carry more RNA than the more widely studied exosomes indicating that LO may be a more robust source of EV-encapsulated miRNA. Gene ontology analysis from miR-1227 targets identified by RNA sequencing from miR-1227 stably expressing PC cells, RISCTRAP Immunoprecipitation of miR-1227 bound mRNA, and in silico miRNA target prediction highlighted several genes related to EV secretion. miR-1227 alters the localization of exosome and LO markers in multiple cancer cell lines, and induces the shedding of LO while inhibiting the shedding of exosomes. Furthermore, miR-1227 induces the migration of poorly migratory cancer cells and increases the expression of tumour supportive cytokines.
Summary/Conclusion: Together these data hint that miR-1227 may promote prostate cancer progression through several mechanisms including alteration of EV shedding.

Funding: 2017–2022 R01CA218526.
2018–2020 Chesapeake Urology Associates Sanford J. Siegel, MD Prostate Cancer Research Scholarship
2018–2020 Luke Wu-Jei Chang Discovery Fund
2016–2019 PI DoD PCRP Award PC150836

OWP1.02=PF11.14

MSC exosome works through a multi-faceted mechanism of action in joint repair

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Introduction: MSC exosome is increasingly accepted as the principal agent that underpins the therapeutic efficacy of mesenchymal stem cell (MSC) in tissue repair. Here, we aim to elucidate the mechanism of action (MoA) of MSC exosome in immunocompetent rat models of osteochondral defect and osteoarthritis (OA).
Methods: Exosomes were purified from conditioned medium of human MSCs by size fractionation. Osteochondral defect creation or anterior cruciate ligament transection to induce OA were performed in 72 adult rats. Thereafter, weekly 100-µl intra-articular injections of 100-µg exosome or PBS vehicle were given. Analysis included weight distribution, histology, immunohistochemistry and cytokine assay. Cellular assays using chondrocytes were performed to determine the exosome-activated cellular processes and signalling pathways.

Results: We observed that exosome-mediated repair of osteochondral defects was characterized by increased cellular infiltration and proliferation, enhanced matrix synthesis, together with a regenerative M2 macrophage phenotype and a reduction in pro-inflammatory cytokines IL-1β and TNF-α. In OA joints, MSC exosome mediated an early suppression of pain and degeneration with reduced inflammation, followed by sustained proliferation and matrix restoration that led to cartilage and subchondral bone regeneration. Using chondrocyte cultures, we could attribute some of these cellular activities during exosome-mediated joint repair to exosomal CD73-mediated adenosine activation of AKT and ERK signalling. These effects were partially abrogated by wortmannin or U0126, which inhibited AKT and ERK phosphorylation, respectively. The role of exosomal CD73 was confirmed using CD73 inhibitor and theophylline that showed inhibition of exosome-induced AKT and ERK phosphorylation.
Summary/Conclusion: Our observations suggest that MSC exosome works through a multi-faceted MoA that involved multiple cellular processes to restore joint homeostasis and promote regeneration.
Funding: National Medical Research Council Singapore (NMRC/CNIG/1168/2017 and NMRC/CIRG/1480/2017).

OWP1.03=PS03.11

Identification of extracellular vesicles as biomarkers for myocardial infraction by flow cytometry and automated data processing

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Introduction: Acute myocardial infarction (AMI) is a major cause of death. To diagnose AMI, measuring troponin concentration is the gold standard. Since troponin is unspecific for AMI, novel biomarkers for AMI are urgently needed. After the onset of AMI, platelets, endothelial cells and blood cells release specific extracellular vesicles (EVs). Our aim is to identify these EVs as biomarkers for AMI diagnosis and treatment monitoring.
Methods: The study was approved by the medical ethics committee. Venous blood was collected 24 h, 72 h and 6 months after AMI from fasting patients (n = 60, 64.5 ± 10.8 years, 68% male) and healthy controls (n = 30, 57.7 ± 6.6 years, 62% male). Flow cytometry (Apogee A60 Micro) was used to determine plasma concentrations of EVs labelled with antibodies for activated platelets (CD61, CD62p; PEVs), endothelial cells (CD146; EEVs) and red blood cells (CD235a; RBC-EVs). Processing of 1,224 flow cytometry data files was performed using in-house developed, automated software (MATLAB R2018a), enabling flow rate stabilization, diameter and refractive index determination, MESF calibration, fluorescent gate determination and statistics reporting.
Results: Between AMI patients and controls, PEV concentrations in plasma were comparable (p = ns), EEV concentrations increased ($p < 0.0001$) and RBC-EV concentrations decreased ($p < 0.0001$). Antiplatelet drug ticagrelor decreased concentrations of PEVs ($p = 0.03$), compared to less potent clopidogrel but did not affect EEVs and RBC-EVs. In turn, concentrations of EEVs, but not PEVs and RBC-EVs, positively correlated with the dose of atorvastatin ($p < 0.001$). The antioxidative β-blocker carvedilol increased concentrations of RBC-EVs, compared to nebivolol ($p = 0.05$) but did not affect PEVs and EEVs.
Summary/Conclusion: Flow cytometry and automated data processing were used to find biomarkers for AMI based on EVs in plasma. During treatment, ticagrelor decreased PEV concentrations, atorvastatin increased EEV concentrations and carvedilol increased RBC-EV concentrations, suggesting that EVs might be used to monitor AMI treatment. AMI patients differed from controls regarding EEV and RBC-EV concentrations, but not PEVs, likely because blood was collected 24 h after the start of antiplatelet therapy. In follow-up studies, it is crucial to collect blood prior to treatment.

OWP1.04=PF11.15

Exosome mediated enhancement of cellular therapy in acute myelogenous leukemia (AML)

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Introduction: Of the AML patients able to tolerate curative therapy with chemotherapy and stem cell transplant many are challenged by treatment related toxicities as well as graft vs. host disease. There is novel work exploring the utility of haploidentical cellular therapy infusion in order to incite purposeful recipient immune response and subsequent cytokine storm to treat refractory AML. Our group has demonstrated the healing potential of bone marrow derived mesenchymal stem cell extracellular vesicles (MSC-

EVs) across multiple disease states, most recently demonstrating the pro-apoptoic signalling imparted by these nanoparticles on nascent leukemic cells *in vivo*; as well as the potentiating effects of MSC-EVs when used as an adjunct to standard cytarabine chemotherapy. We have also shown the protective role of hMSC EV on radiated BM and stem cell recovery.

Methods: Kasumi AML cells lines were seeded with MSC-derived EVs. Vesicles were isolated using an established differential centrifugation technique, and were co-cultured with Kasumi cells for various time points. To study cellular viability, we used a fluorescence-based method for quantifying viable cells.

We also explored various modes of death EVs may illicit via a tri-dye Abcam assay designed to simultaneously monitor apoptotic, necrotic and healthy cells. Both assays were used to measure viability and apoptosis in similar experiments employing cytarabine

Results: AML cell Proliferation Decreased after 1–6 days of co-culture with hMSC-derived EVs.

Apoptosis is the primary mode of death induced.

AML cell proliferation decreased synergistic after 1–6 days of co-culture with hMSC-derived EVs ± Cytarabine.

Summary/Conclusion: MSCs inhibits the proliferation of the AML cell line *in vitro* and work synergistically with cytarabine chemotherapy to promote apoptotic death in AML cell lines. Our prior work has shown that MSC-EVs can abate the effects of toxic chemo/radiation and serve to protect stem cell allowing for quicker recover in cell blood counts.

Based on the innate ability of MSC-EV to directly alter the cellular machinery of abnormal leukemic cell and of nascent immune cells our corollary hypothesis is that BM-derived MSC-EVs may serve as suitable alternative to conditioning chemo/radiation in the AML setting and will enhance the effects seen by cellular therapy infusion.

Funding: t32.

OWP1.05=PF12.09

Extracellular vesicles derived from amniotic fluid stem cells rescue impaired foetal lung development via the release of microRNAs

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Introduction: Incomplete lung development, also known as pulmonary hypoplasia (PH), is a recognized cause of neonatal death. To date, there is no effective treatment that promotes foetal lung growth and maturation. Herein, we describe a stem cell-based approach that enhances foetal lung development via the administration of extracellular vesicles (EVs) derived from amniotic fluid stem cells (AFSCs) in rat models of PH. Moreover, we report the microRNAs present in AFSC-EVs that are responsible for these beneficial effects.

Methods: AFSC-EVs were isolated by ultracentrifugation from conditioned medium (CM) of c-Kit+ rat AFSC that were grown in exosome-depleted FBS for 18h. AFSC-EVs were assessed for size (nanoparticle tracking analysis), morphology (TEM), and expression of CD63, Hsp70, Flo-1 and TSG101 (Western).

Ex vivo: Pregnant dams were gavaged nitrofen at E9.5 to induce foetal PH. At E14.5, foetal lungs were harvested, and incubated with culture medium alone, AFSC-CM, or AFSC-EVs. Foetal lungs from untreated dams served as control. Lungs were compared for terminal bud density and surface area at 72 h, by two independent investigators.

In vitro: Foetal rat lung organoids were generated with epithelial cells from normal and hypoplastic lungs. Organoids were cultured for 10 days in either medium alone or medium supplemented with AFSC-EVs. Lung organoids from untreated normal pups served as control. Organoids were assessed for proliferation (Ki67) and markers of epithelial cell differentiation via immunofluorescence.

RNA-sequencing: RNA was isolated using SeraMir, constructed into libraries (CleanTag Small RNA) and sequenced on NextSeq High Output single-end sequencing run.

Results: Administration of AFSC-EVs increased terminal bud density and surface area of lung explants back to control levels and promoted lung epithelial cell differentiation in lung organoids (increased SPC and CC10 expression). AFSC-EVs contain 901 microRNAs, some of which are crucial for foetal lung development, such as miR17 ~ 92 cluster.

Summary/Conclusion: Administration of AFSC-EVs rescues impaired foetal lung development in experimental models of PH. AFSC-EV regenerative ability is exerted via the release of miRNAs some of which regulate genes involved in foetal lung development. AFSC-EVs represent a promising therapeutic strategy for PH in foetuses.

Funding: CIHR-SickKids Foundation.

OWP1.06=PS01.11

Extracellular vesicles from Fat-laden hypoxic hepatocytes activates pro-fibrogenic signals in Hepatic Stellate Cells

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Introduction/Background: Transition from isolated steatosis to non-alcoholic steatohepatitis is a key issue in non-alcoholic fatty liver disease (NAFLD). Recent observations in patients with obstructive sleep apnoea syndrome (OSAS), suggest that hypoxia may contribute to disease progression mainly through activation of hypoxia inducible factor 1α (HIF-1α)-related pathways. Release of extracellular vesicles (EV) by injured hepatocytes may be involved in NAFLD progression.
Aim: to explore whether hypoxia modulates the release of EV from free fatty acid (FFA)-exposed hepatocytes and assess cellular crosstalk between hepatocytes and LX-2 cells (human hepatic stellate cell line).
Methods: HepG2 cells were treated with FFAs (250 µM palmitic acid + 500 µM oleic acid) and chemical hypoxia (CH) was induced with Cobalt (II) Chloride, which is an inducer of HIF-1α. Induction of CH was confirmed by Western blot (WB) of HIF-1α. EV isolation and quantification was performed by ultracentrifugation and nanoparticle tracking analysis respectively. EV characterization was performed by electron microscopy and WB of CD-81 marker. LX-2 cells were treated with 15 µg/ml of EV from hepatocytes obtained from different groups and markers of pro-fibrogenic signalling were determined by quantitative PCR (qPCR), WB and immunofluorescence (IF).
Results: FFA and CH-treatment of HepG2 cells increased gene expression of IL-1β and TGF-β1 in HepG2 cells and increased the release of EV compared to non-treated HepG2 cells. Treatment of LX-2 cells with EV from FFA-treated hypoxic HepG2 cells increased gene expression of TGF-β1, CTGF, α-SMA and Collagen1A1 compared to LX-2 cells treated with EV from non-treated hepatocytes or LX-2 cells exposed to EV-free supernatant from FFA-treated hypoxic HepG2 cells. Moreover, EV from FFA-treated hypoxic HepG2 cells increased Collagen1A1 and α-SMA protein levels.
Summary/Conclusion: CH promotes EV release from HepG2 cells. EV from hypoxic FFA-treated HepG2 cells evoke pro-fibrotic responses in LX-2 cells. Further genomic and proteomic characterization of EV released by steatotic cells under hypoxia are necessary to further delineate their role in the crosstalk between hepatocytes and stellate cells in the setting of NAFLD and OSAS.
Funding: FONDECYT 1150327–1150311.

OWP1.07=PS08.07

Exploration of the surface modification of outer membrane vesicles

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Introduction: Introducing bacteria-binding small molecules to the surface of outer membrane vesicles (OMVs) could greatly improve their potential for antimicrobial drug delivery too difficult to treat bacteria. Among the small number of studies on surface modification of OMVs, very few deal with small molecules. The aim of the present study is to evaluate different methods of introducing bacteria specific targeting moieties to OMVs. We assessed the modification of surface proteins using N-hydroxysuccinimide (NHS) esters, well established for mammalian extracellular vesicles (EVs), cholesterol insertion, mainly applied for liposomes, and the novel application of diazo-transfer followed by click-chemistry.
Methods: OMVs were obtained from model myxobacteria by differential ultracentrifugation (UC) followed by size-exclusion chromatography (SEC). For cholesterol insertion and NHS ester-modification, purified OMVs were incubated with either cholesteryl PEG 2,000 FITC or sulfo cyanine7 NHS ester. For diazo transfer the pellet after UC was incubated with a diazo transfer agent and the OMVs subsequently conjugated with DBCO-AF594. Unincorporated dye was removed by SEC. Liposomes were composed of DMPC and DPPC in 2:3 molar ratio. Results represent correlated fluorescence intensity and particle number.
Results: Treatment with sulfo cyanine7 NHS ester led to the modification with 547 ± 163 molecules per OMVs, compared to 18 ± 1 for the control using sulfo cyanine7 acid. Cholesterol insertion introduced 4 ± 1 molecules per OMV, compared to 101 ± 23 for liposomes. First results for the diazo-transfer showed 71 dye-molecules per OMV, with 32 for the control.
Summary/Conclusion: Of the three methods, NHS ester-modification displayed the highest efficiency, similar to published results for mammalian EVs. In comparison, diazo transfer only yielded ~13% of the dye-molecules per particle. However, there are still many parameters to be optimized for this method, including OMV concentration and incubation period. Cholesterol insertion was unsuccessful for OMVs,

probably owing to their membrane structure. In this study, we aim to get important insights into the modification of OMVs for bacterial targeting and EV-surface engineering in general.

Funding: This project was funded by Studienstiftung des Deutschen Volkes and Bundesministerium fuer Bildung und Forschung.

OWP1.08=LBT02.03

Isolation of neuron-specific extracellular vesicles

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Introduction: Human biological fluids contain extracellular vesicles (EVs) from different cell types. It would be incredibly useful to be able to isolate EVs that originated from specific cell types for diagnostic purposes as a way to gain molecular information (RNA, protein) from inaccessible cell types non-invasively.

Methods: We have developed a general framework for identifying EV surface markers that can be used for immuno-isolation of cell type specific EVs. As a proof of principle, we have applied this framework to the isolation of neuron-derived EVs from human cerebrospinal fluid or plasma. In addition to the computational analysis, we have developed an *in-vitro* system of human neurons differentiated from human induced pluripotent (iPS) cells. We performed mass spectrometry on EVs isolated from these neurons to identify neuron-specific proteins. We also used this system to develop a robust immune-isolation method for neuron EV markers.

Results: We have characterized the proteins present in neuron exosomes by mass spectrometry and then used computational analysis of published gene expression and proteomics data to come up with a list of candidate neuron-specific EV markers. After developing methods for immuno-isolation of neuron EVs with these markers, we applied our methods to human cerebrospinal fluid and plasma.

Summary/conclusion: We have developed a framework for the isolation of cell type specific EVs through the combination of an experimental *in vitro* system and computational analysis of gene expression and proteomics data. We have applied this framework to the isolation of neuron-specific EVs in human biological fluids. We envision these methods being broadly applicable to the development of novel diagnostic biomarkers for a variety of diseases.

OWP1.09=LBT01.01

Coagulation influences properties of extracellular vesicles isolated from autologous blood derived products

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Introduction: Platelet rich plasma (PRP) is the most commonly used blood derivative in clinics due to its high concentration of platelets and perceived high growth factor levels. Drawbacks of using PRP are discrepancies among preparation protocols and the presence of cells (platelets, leucocytes) which can evoke cellular processes (e.g. inflammation) when injected into the host. One possibility is to isolate only the active components of blood derivatives which may overcome this problem. In the current study, we focused on extracellular vesicles (EVs) isolated from two autologous blood derivatives, PRP and hyperacute serum and investigated whether the clotting cascade influences EV properties.

Methods: EVs were isolated from citrate-anticoagulated PRP (CPRP) and hyperacute serum using differential ultracentrifugation followed by a size exclusion chromatography. Particle concentration and size were determined by nanoparticle tracking analysis (NTA). Cryo-electronmicrosopy was performed to visualize isolated EVs. Expression of miRNAs transported within EVs as well as in their respective input material was analysed by qPCR.

Results: NTA revealed higher particle concentrations and bigger sized EVs within CPRP compared to hyperacute serum. These findings were confirmed by cryo-electronmicroscopy. Profound differences were detected regarding miRNA expression between the two blood derivatives. In total, 126 miRNAs were identified which were expressed both in input material as well as in the corresponding EVs. The correlation between miRNAs in EVs and input material was higher in CPRP compared to hyperacute serum meaning that in hyperacute serum miRNAs were identified which were higher expressed in EVs than in the corresponding input material.

Summary/conclusion: EVs from autologous blood products represent a novel and cell-free regeneration approach. We observed that the clotting cascade (plasma versus serum) has an influence on concentration, size and miRNA expression patterns of EVs. These differences might have an impact on the biological mode of action of blood-derived products used in clinics.

Funding: Financial support was received from the European Fund for Regional Development (EFRE) and the Science Fund of Lower Austria. miRNA expression analysis was performed by TAmiRNA GmbH. Cryo-electronmicroscopy was conducted at the Core Facility of the Vienna Bio-Center.

OWP1.10=LBF02.01

Type-2 transglutaminase affects calcium homeostasis in neurons and is released in association with astrocytes-derived exosomes

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Introduction: Type-2 transglutaminase (TG2) has been linked to calcium (Ca2+) dysregulation in conditions such as neurodegeneration. Recent evidences suggest that extracellular vesicles (EVs) contribute to the onset and progression of neurological diseases, and we have recently shown that TG2 is a cargo of EVs in biological fluids (Furini et al., 2018). Here, we hypothesise that TG2 could be released by EVs, interact with neurons and affect neuronal Ca2+ homeostasis.

Methods: Primary hippocampal neurons were established from E18 rat embryos. Extracellular TG2 was modulated in neurons either by lipofectamine transfection of a TG2-EGFP construct or by addition of purified TG2. Intracellular Ca2+ concentration ([Ca2+]i) was assessed by live imaging in fura-2/AM-loaded neurons. EVs were isolated from primary astrocytes (60 DIV) by serial centrifugation, characterised by western blotting (flotillin-2 and alix) and nanoparticle tracking analysis (ZetaView). Experiments to assess TG2 influence on exosomes-to-neural cells interactions, using a Renilla sensor based on miR-146a-5p-transfer, are still ongoing.

Results: Increase of extracellular TG2 levels in neurons induced an influx of extracellular Ca2+ ions, leading to a significant raise in basal [Ca2+]i both in normal conditions (ΔF340/380 = 0.126 ± 0.014; N = 23; p < 10–5) and with inhibited synaptic transmission (tetrodotoxin) (ΔF340/380 = 0.058 ± 0.005; N = 33; p < 10–5). Nifedipine, a blocker of L-type voltage-operated Ca2+ channels (VOCCs), partially prevented TG2-dependent Ca2+ response (average inhibition 36%; N = 21; p < 10–5), suggesting that Ca2+ influx may occur through L-type VOCCs. To identify the source of extracellular TG2, we analysed EVs isolated from rat primary astrocytes, previously reported to release TG2 into the matrix especially in inflammatory conditions. TG2 was detected in astrocytic exosomes (but not in ectosomes) only upon LPS stimulus, and not in the EVs-free medium, suggesting that TG2 is a cargo of exosomes during neuroinflammation.

Summary/conclusion: TG2 is externalised through astrocyte-derived exosomes upon neuroinflammatory stimuli. Extracellular TG2 mediates the opening of L-type VOCCs in neurons and sets basal [Ca2+]i at higher levels, which could have a significant impact on neuronal activity in neuroinflammation.

Funding: John Turland PhD bursary (NTU) and IBRO travel fund.

OWP1.11=LBT01.02

Ev-avogadro project: towards a liposomal concentration standard for extracellular vesicle research

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Introduction: There is an unmet need for standardization of concentration measurements in the field of extracellular vesicles (EVs). Liposomes may serve an ideal reference system for EVs, but the determination of the number concentration of liposomes from first principles was not attempted so far. Inspired by the International Avogadro project, we aimed to determine the concentration of liposomes with well-defined size and composition via counting the number of phospholipid molecules in these "nanospheres".

Methods: Liposomes composed of phosphocholine and phosphoglycerol were prepared by the extrusion method. Wide-angle X-ray scattering (WAXS) was used to determine the area-per-lipid value. The size distribution of the liposomes was determined by microfluidic resistive pulse sensing (MRPS) and freeze-fracture combined TEM. Small-angle X-ray scattering (SAXS), differential scanning calorimetry (DSC) and infrared spectroscopy (IR) were used to prove the unilamellarity, the ideal miscibility of the lipids and the ordered packing of the hydrocarbon chains of the lipids, respectively. Concentration of the lipids was determined by liquid chromatography–mass spectrometry (LC-MS).

Results: The prepared liposomes proved to be unilamellar with narrow size distribution (83 nm avg.), as obtained by MRPS and TEM. DSC and IR measurements confirmed that the phospholipid bilayer of these liposomes is in the liquid-ordered phase, hence the area-per-lipid of 0.41 nm^2 was determined from WAXS measurements. Using the concentration of

phospholipids from LC-MS measurements, the number concentration of liposomes was determined (8E+13 1/mL).

Summary/conclusion: Liposomes containing saturated phospholipids are in the liquid-ordered phase, which can be utilized to determine the area-per-lipid using WAXS. This value, together with the independently determined size, and lipid concentration can be used to calculate the number concentration of liposomes. As the light scattering properties of liposomes matches that of EVs, liposome-based standards for optical measurements of EVs can be obtained with the presented techniques.

Funding: This work was supported under grant numbers PD 121326 and NVKP_16-1-2016-0007 by NKFIH (Hungary). ZV was supported by the János Bolyai Research Fellowship.

OWP1.12=LBF02.02

Plasma exosomes regulate proliferation and migration of vascular smooth muscle cells

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Introduction: We previously reported that systolic blood pressure in spontaneously hypertensive rats, an animal model of essential hypertension, was partly modulated by circulating exosomes (*BBRC* 2018). Vascular wall remodelling regulated by proliferation and migration of vascular smooth muscle cells (VSMCs) mediates development of hypertension. We aimed to clarify the effects of plasma exosomes derived from SHR and control Wistar Kyoto Rats (WKY) on proliferation and migration of VSMCs.

Methods: Exosomes were isolated from rat plasma by an ultracentrifuge method, and identified through measurement of particle size distribution by a tunable resistance pulse sensing. For exploring exosome internalization in VSMCs, the isolated exosomes were labelled with PKH67 dye and observed by a fluorescence microscopy. Proliferation and migration of SMCs were determined by a bromodeoxyuridine incorporation and Boyden chamber assay, respectively. Actin cytoskeleton was visualized by a rhodamine-phalloidin staining. Expression of protein and microRNA in exosomes was determined by Western blotting and microarray, respectively.

Results: There was no difference in size and concentration of plasma exosomes between WKY and SHR. Exosomes were incorporated into VSMCs, while the internalization of SHR exosomes was significantly lower than WKY exosomes. Both WKY and SHR exosomes similarly stimulated proliferation, migration and cytoskeletal changes such as formation of filopodia and lamellipodia in VSMCs. Heparin, an inhibitor of exosome internalization, completely blocked the migration and proliferation. Protein expression of CD9 and CD63, an exosomal marker, was significantly higher in exosomes from WKY than SHR. The expression of several microRNAs in SHR exosomes changed compared with WKY exosomes.

Summary/conclusion: These results suggest that plasma exosomes play physiological, but not pathological, role on VSMCs irrespective of their origin (normotensive or hypertensive rats). Further research is required for determining whether the changes in molecular profiles of circulating exosomes mediate the development of high blood pressure in SHR.

OWP1.13=LBF01.02

Colorectal cancer cell-derived exosome enhances microenvironmental angiogenesis through modulation of intracellular metabolism

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Introduction: For improvement of prognosis of colorectal cancer (CRC), detection at an earlier stage of CRC is essential. Exosomes are nanovesicles secreted from plasma membrane, and have potential to be served as biomarker carriers. In this study, we performed proteomic profiling of exosomes secreted from viable CRC tissues.

Methods: To identify early detection biomarkers for CRC, we performed comprehensive proteome analysis of tissue-exudative extracellular vesicles (Te-EVs), which were obtained from culture media of freshly resected viable CRC tissue or adjacent normal mucosa (n = 17). Among the identified Te-EV proteins, we narrowed down the biomarker candidate by selecting proteins which are statistically upregulated ($p < .05$, fold change > 5.0) in Te-EVs from CRC tissues than those from adjacent normal tissues. Then we performed functional analysis of the biomarker candidate specifically.

Results: Comprehensive LC/MS analysis identified 6,149 Te-EV proteins, in which 641 proteins showed significant upregulation in Te-EVs from CRC tissues

($p < .05$, fold change > 5. 0) compared to those from adjacent normal mucosa. We focused especially on GAM ($p = 7.0 \times 10–5$, fold change = 7.4) as a novel biomarker candidate. GAM protein was significantly overexpressed in CRC tissues compared with adjacent normal mucosa. In EV-sandwich ELISA assay, the expression level of GAM on plasma EVs from CRC patients was significantly higher than that from healthy donors in EV-sandwich ELISA assay ($n = 133$, $p = 4.0 \times 10^{-7}$). In addition, the uptake of GAM-overexpressing EVs enhanced vascular endothelial cell growth and angiogenesis through modulation of nitric oxide metabolism.

Summary/conclusion: EV-GAM might have great potential as a target for both CRC diagnosis and therapy. Our strategy for identification of exosomal biomarker by proteomic profiling of Te-EV proteins can be applied to other cancers.

OWP1.14=LBS02.01

Annexin V binding modulates the response of macrophages to mesenchymal stromal cell-derived extracellular vesicles

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Introduction: We have previously shown that Annexin a5 (An5) binding to mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) enhances the anti-inflammatory properties of these nanoparticles in an animal model of colitis. However, the mechanisms underlying these effects are unknown. Here, we investigated the immunoregulatory effect of MSC-EVs with and without An5 binding on activated macrophages *in vitro*.

Methods: Macrophages were isolated from mouse bone marrow and activated by INFgamma and LPS. Clinical grade Wharton Jelly-derived MSC-EVs were obtained from The Cell Factory (Esperite NV, Niel, Belgium) and quantified by Resistive Pulse Sensing analysis. 5,0E+05 macrophages were incubated with PBS (vehicle only, control, group 1) 5,0E+08 MSC-EVs (group 2), 5,0E+08 MSC-EVs added with 2 ug An5 (group 3) or with 2 ug free An5 (group 4). After 24 h, the cells were analysed by flow cytometry and RNA was extracted for RT-PCR analysis.

Results: Incubation with MSC-EVs significantly increased only the expression of IL-10 in IFN-gamma/LPS-activated macrophages. Incubation with An5-MSC-EVs resulted in a significant induction in the expression of both pro- and anti-inflammatory cytokines, including TNF-alfa, IL-1Beta, IL-6, IL-10 and TGFbeta1. Incubation with free An5 induced only pro-inflammatory cytokines without affecting IL-10 and TGFbeta1 expression. The iNOS2/Arg1 ratio was reduced in both EV-treated groups, indicating a shift from M1 to M2 polarization.

Summary/conclusion: In conclusion, both MSC-EVs and An5-MSC-EVs shift the macrophage phenotype from M1 to M2. The combined induction of TGFbeta1 and IL-10, observed only in An5-MSC-EV-stimulated macrophages, might be related to the immune-modulating characteristics of these modified EVs that contribute to the therapeutic effects observed *in vivo*.

Funding: The BROAD MEDICAL RESEARCH PROGRAM AT CCFA supported this work

OWP1.15=LBS03.01

Membrane-radiolabelled exosomes for comparative biodistribution analysis in immunocompetent and immunodeficient mice – A novel and universal approach

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Introduction: Exosomes have gained interest as novel drug nanocarriers due to their biological origin and role in intercellular biomolecule delivery. In-depth knowledge of their *in vivo* biodistribution is therefore essential. This work aimed to develop a reliable and universal method to radiolabel exosomes to study *in vivo* biodistribution in mice.

Methods: Melanoma (B16F10 cells)-derived exosomes (ExoB16) were isolated and characterised for size, yield, purity, exosomal markers and morphology using Nanoparticle Tracking Analysis (NTA), protein measurements, flow cytometry and electron microscopy. Two radiolabelling approaches were explored – intraluminal labelling (111Indium entrapment via tropolone shuttling); and membrane labelling (111Indium chelation by covalently attached bifunctional chelator). Labelling efficiency and stability was assessed by gel filtration and thin layer chromatography. Melanoma-bearing immunocompetent (C57BL/6) and immunodeficient (NSG) mice were injected intravenously with radiolabelled ExoB16 (1x1011 particles) followed by

metabolic cages study, whole body SPECT-CT imaging and *ex vivo* gamma counting at 1, 4 and 24 h post-injection.

Results: Membrane-labelled ExoB16 (ML-ExoB16) showed superior radiolabelling efficiency and radiochemical stability compared to intraluminal-labelled ExoB16 (IL-ExoB16). Both IL- and ML-ExoB16 showed prominent accumulation in liver and spleen. IL-ExoB16 showed higher tumour accumulation than ML- ExoB16 (6.7% and 0.6% ID/g tissue, respectively), with the former showing similar value as its free tracer ([111]Trop). The superior stability of the membrane-labelling approach rendered its result more reliable and was used to compare ExoB16 biodistribution in melanoma-bearing immunocompromised (NSG) mice. Similar biodistribution profile was observed in both C57BL/6 and NSG mice, where prominent accumulation was seen in liver and spleen, apart from the lower tumour accumulation observed in the NSG mice.

Summary/conclusion: Membrane radiolabelling of exosomes is a reliable approach that allows for both live imaging and quantitative biodistribution studies to be performed on potentially all exosome types without engineering parent cells.

Oral with Poster Session 2
Chairs: Kazunari Akiyoshi; Muller Fabbri
Location: Level B1, Lecture Room

13:30–15:00

OWP2.01=PS08.08

Identification of common EV markers in plasma using high-resolution flow cytometry

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Introduction: Recent advancements in flow cytometry (FCM) have led to the development of high-resolution FCMs dedicated to the analysis of small particles (hFCM). hFCM studies have predominantly focused on the analysis of EVs expressing phosphatidylserine (PS). PS is enriched in microvesicles (MVs), wherein it is involved in lipid rearrangements responsible for MV budding. While PS also is expressed on exosomes, it is unknown whether it can be used as a universal marker for smaller EVs. In this study, we attempted to characterize proteins enriched in smaller EVs (CD9, CD63, CD81 and ADAM 10) and the relative co-expression of PS with each of these markers.
Methods: FCM analysis was performed on an Apogee A60 Micro-PLUS. In brief, platelet-poor plasma (PPP) from healthy individuals was stained with lactadherin-FITC (PS+) and one of several EV surface markers enriched in smaller EVs. To evaluate the precise differences in PS and specific EV marker expression, the analysis was performed twice, (1) triggering on lactadherin and (2) each EV marker (CD9-PE, CD81-PE, CD63-PE, ADAM10-PE), separately. All antibodies were matched with appropriate isotope controls and centrifuged at 17,000*g* for 10 min prior to antibody labelling. EVs were defined as lactadherin or EV surface marker positive events ≤1000 nm.
Results: Initial results indicate that CD9 is highly expressed on EVs and is not universally associated to PS. Triggering on PS revealed that 34.7% of all events were CD9 positive (CD9+|PS+). Conversely, triggering on CD9 resulted in a 2.1-fold increase in total events, where 17.0% of events were PS+ (CD9+|PS+). Inferring size from silica nanospheres, it appeared that populations containing CD9 (CD9+|PS+ and CD9+|PS-) were smaller (94.4–99.7% <180 nm) compared to populations that did not (PS+|CD9-; 85.6% <180 nm & 95.2% <300 nm). Interestingly, we did not detect CD81, CD63 or ADAM10 on EVs. We hypothesize that this is due to a low abundance of these markers in PPP from healthy individuals.
Summary/Conclusion: Our findings demonstrate that hFCM can be used for the characterization of smaller EVs in PPP. Furthermore, we find that CD9+EVs do not universally express PS. From this point on, we plan to study enrichment of these EV phenotypes following a number of EV purification protocols, and determine whether EV isolation enable a more extensive characterization of smaller EVs.

OWP2.02=PS08.09

Software to automate calibration and processing of flow cytometry data in clinical studies

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Introduction: In search of new biomarkers, flow cytometers are used in clinical studies to measure the concentration of specific extracellular vesicles (EVs). Flow cytometers measure light scattering and fluorescence of single EVs in a fluid stream. However, to realize data interpretation and comparison, light scattering and fluorescence signals and the flow rate require calibration. Moreover, flow cytometers generate large datasets. For example, a clinical study involving 60 patients, 30 controls, and 8 antibody labels covers 1224 data files, >33 gigabytes of data and >0.3 billion events. To manually calibrate and analyse such a dataset would take days if not weeks and is prone to human mistakes. Therefore, an urgent need exists for software to automate calibration and processing of flow cytometry data.
Methods: We have developed software (MATLAB R2018a) to automatically process multiple .fcs files and (1) relate two scatter signals to the diameter in nm and refractive index (RI) of EVs, (2) express

fluorescence signals in terms of molecules of equivalent soluble fluorochrome, (3) export calibrated channels to new .fcs files, (4) recognize unstable flow rates, (5) determine fluorescence thresholds, (6) apply gates, (7) create PDFs with scatter plots and (8) report statistics. We are using clinical studies to validate and apply the software.

Results: Compared to manual thresholding, automatic thresholding results in a systematic decrease in counts of 10% and a maximum difference of 14% (n = 5). Using a high-end laptop, data processing takes typically a minute or several seconds per .fcs file with or without PDF reporting, respectively. Flow rate monitoring is useful for 61% of the data. The platelet marker CD61 stains 7% of the events with an RI >1.42, which are lipoproteins, and the concentration of these lipoproteins differed 4000-fold between individuals.

Summary/Conclusion: We have developed software to automate calibration and processing of flow cytometry data in clinical studies, thereby reducing analyses time, preventing human mistakes and providing new insights. For example, non-specific labelling of antibodies to lipoproteins together with variations in lipoprotein concentrations emphasize the relevance of fasting before venipuncture. Our next step is to extend the software with machine learning.

Funding: NWO-TTW VENI 15924.

OWP2.03=PS08.10

Conventional, high-resolution and imaging flow cytometry: potentials, pitfalls and solutions for EV characterization

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Introduction: Flow cytometry (FCM) has long been a preferred method for characterizing EVs, however their small size have limited the applicability of conventional FCM to some extent. Thus, high-resolution and imaging FCMs have been developed but not yet systematically evaluated. The aim of this presentation is to describe the applicability of high-resolution and imaging FCM in the context of EV characterization and the most significant pitfalls potentially influencing data interpretation.

Methods: (1) First, we present a side-by-side comparison of three different cytometry platforms on characterising EVs from blood plasma regarding sensitivity, resolution and reproducibility: a conventional FCM, a high-resolution FCM and an imaging FCM. (2) Next, we demonstrate how different pitfalls can influence the interpretation of results on the different cytometry platforms. (3) Finally, we propose controls, solutions or workarounds for understanding and limiting the influence of each of these pitfalls.

Results: (1) High-resolution FCM and imaging FCM displayed greater sensitivity and resolution compared to conventional FCM when measuring a mixture of nanospheres. Equally, both methods could detect larger concentrations of specific EV phenotypes than conventional FCM, where imaging FCM outperformed high-resolution FCM. Within day variability (n = 20 aliquots) was similar for conventional and high-resolution FCM, while imaging FCM had a markedly larger variability. Between day variability ($n = 5 \times 5$ aliquots) was similar for all three platforms. (2) The three most substantial pitfalls variably influencing interpretation of results on the three platforms are non-specific binding of labels, antibody aggregates, and entities in the sample (i.e. lipoproteins) binding EV-defining dyes. (3) The most important strategies for circumventing these pitfalls are stringent matching, gating and comparison of antibodies and isotype controls, high-speed centrifugation of antibodies and labels prior to staining, and the use and interpretation of stained buffer controls and detergent treated samples.

Summary/Conclusion: High-resolution and imaging FCM hold great potential for EV characterization. However, increased sensitivity also leads to new artefacts and pitfalls. The solutions proposed in this presentation provide useful strategies for circumventing these.

OWP2.04=PS08.11

Convolutional neural networks for classification of tumour derived extracellular vesicles

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Introduction: Raman spectroscopy probes molecular vibration and thus reveals chemical information of a sample without labelling. This optical technique can be used to study the chemical composition of diverse extracellular vesicles (EVs) subtypes. EVs have a complex chemical structure and heterogeneous nature so that we need a smart way to analyse/classify the obtained Raman spectra. Machine learning (ML) can be a solution for this problem. ML is a widely used strategy in the field of computer vision. It is used for recognizing patterns and images as well as classifying data. In this research, we applied ML to classify the EVs' Raman spectra.

Methods: With Raman optical tweezers, we obtained Raman spectra from four EV subtypes – red blood cell, platelet PC3 and LNCaP – derived EVs. To classify them by their origin, we used a convolutional neural network (CNN). We adapted the CNN to one-dimensional spectral data for this application.

The ML algorithm is a data hungry model. The model requires a lot of training data for accurate prediction. To further increase our substantial dataset, we performed data augmentation by adding randomly generated Gaussian white noise.

The model has three convolutional layers and fully connected layers with five hidden layers. The Leaky rectified linear unit and the hyperbolic tangent are used as activation functions for the convolutional layer and fully connected layer, respectively.

Results: In previous research, we classified EV Raman spectra using principal component analysis (PCA). PCA was not able to classify raw Raman data, but it can classify preprocessed data. CNN can classify both raw and preprocessed data with an accuracy of 93% or higher. It allows to skip the data preprocessing and avoids artefacts and (unintentional) data biasing by data processing.

Summary/Conclusion: We performed Raman experiments on four different EV subtypes. Because of its complexity, we applied a ML technique to classify EV spectra by their cellular origin. As a result of this approach, we were able to classify EVs by cellular origin with a classification accuracy of 93%.

Funding: This work is part of the research programme [Cancer-ID] with project number [14197] which is financed by the Netherlands Organization for Scientific Research (NWO).

OWP2.05=PS08.12

Microfluidic electrochemical aptasensor for detection of breast cancer-derived exosomes in biofluids

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Introduction: Exosomes are nano-sized extracellular vesicles, which are emerging as potential noninvasive biomarkers for early diagnosis of cancer. However, the small size and heterogeneity of the exosomes remain significant challenges to their quantification in the biofluids. In the present research, a microfluidic electrochemical biosensing system (MEBS) is introduced to detect ultra-low levels of breast cancer cell-derived exosomes (BCE).

Methods: Fabrication procedure of MEBS comprises three main steps: first, biosensing surface was prepared by immobilizing EPCAM binding aptamer (EBA) on a nanostructured carbon electrode. The nanostructured surface (NS) consists of 2-D nanomaterials including MoS2 nano-sheets, graphene nano-platelets, and a well-ordered layer of electrodeposited gold nanoparticles. The NS was well characterized with FESEM and EDX. FESEM analysis showed a well-ordered gold nano-structuring for 50 nM of gold solution. Furthermore, EDAX analysis confirmed >60% coverage of gold nanoparticles on NS compared to bare carbon electrode. At the second step, a herringbone structured microfluidic channel, which is able to enrich BCE was designed and fabricated. Finally, microfluidic channel was integrated to biosensing surface. Different concentrations of exosome solutions was introduced and enriched to biosensing surface (SPCE/NS/GNP/EBA) using microchannel. After capturing BCEs on the sensing surface a secondary aptamer labelled with silver nanoparticles (SNPs) as redox reporter was introduced to the sensing surface.

Results: Direct electro-oxidation of SNPs was monitored as analytical signal. The unique design of microchannel in combining with high specific interaction between BCE and EBA provided a high sensitive detection of BCE as low as ~100 exosomes/μL.

Summary/Conclusion: The unique design of MEBS provides a highly sensitive accurate platform for detection of ultra-low levels of cancer-derived exosomes. This tool holds great potential for early cancer diagnosis in clinical applications.

OWP2.06=PS08.13

A software suite allowing standardized analysis and reporting of fluorescent and scatter measurements from flow cytometers

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Introduction: Single vesicle analysis using flow cytometry is an extremely powerful technique to allow identification of unique proteins in biological samples, as well as enumerating the changes in concentrations. While small particle analysis (for viruses and large microparticles) using flow cytometry has been conducted for several decades, there is no comprehensive method for standardization of such studies. Therefore, we developed a suite of flow cytometry post-acquisition analysis software (FCM_{PASS}) tools that enable the conversion of scatter and fluorescent axes to standardized units using appropriate controls, writing standardized

units to .fcs files for sharing upon publication with open repositories, and exporting templates of obtained data.

Methods: Standalone software packages for scatter and fluorescent standardization were built using MATLAB. The scatter software is based upon Mie modelling and is capable of predicting the optical collection angle of the instrumentation and reporting the Mie modelling criteria in a standardized way, making it possible to reproduce the models and flow cytometry settings. Fluorescent standardization data uses least-squares linear regression to enable conversions of arbitrary unit scales to molecules of equivalent soluble fluorophore (MESF) using MESF calibration beads.

Results: The FCM_{PASS} software converts arbitrary fluorescence units to MESF units and writes them to data files for clearer reporting and sharing of data. FCM_{PASS} also converts arbitrary scatter units to a measurement of scattering cross-section using modelling software that predicts the collection angle of the instruments and normalizes the data automatically.

Summary/Conclusion: Utilization of our FCM_{PASS} software can help the EV flow cytometry more easily implement standardization into their experimental analysis and the use of the output templates can make reporting more consistent. While currently available MESF controls can be further optimized for small particles, we believe their utilization along with the other controls, can bring a new era to the reporting of EV research using flow cytometry. This will be particularly useful for future comparison and validation of translational studies and will enable better understanding and utilization of EVs across a broad range of disciplines.

OWP2.07=PF05.08

Biogenesis of JC polyomavirus associated extracellular vesicles depends on neutral sphingomyelinase 2

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Introduction: JC polyomavirus is a non-enveloped virus that causes progressive multifocal leukoencephalopathy (PML) in immunocompromised patients. JCPyV infects cells by first binding to the major attachment receptor lactoseries tetrasaccharide C (LSTc), followed by the serotonin receptor 5-hydroxytryptamine type 2 required for entry. In PML, JCPyV undergoes lytic infection in oligodendrocytes and astrocytes, both of which have been shown to lack LSTc. Further, deep sequencing has shown that viral quasispecies existing in PML patients contain mutations in the sialic acid binding pocket of the major viral capsid protein, rendering these virions incapable of binding LSTc. We have recently demonstrated that JCPyV is packaged into extracellular vesicles (EVs) that can spread the virus, potentially overcoming this paradox. Here, we begin to characterize the biogenesis of this EV-virus association by examining endosomal sorting complexes required for transport (ESCRT) proteins and neutral sphingomyelinase 2 (nSMase2).

Methods: Cambinol was used to specifically target nSMase2 activity. Knockdown cell lines were created with shRNA targeted against ALIX, TSG101 or SMPD3. SMPD3 was also targeted using CRISPR/Cas9 genetic knockout in separate cell lines. Knockdown was confirmed by qPCR and/or Western blot, and knockout by next generation sequencing. EV were concentrated by differential centrifugation and evaluated by transmission electron microscopy, Western blot, nanoparticle tracking analysis, infection and qPCR for protected viral genomes. Infection was scored by immunofluorescence analysis with antibodies against the major viral capsid protein VP1.

Results: We found that depletion of nSMase2 by cambinol, genetic knockdown or knockout caused a reduction in spread of JCPyV over time. Knockdown and knockout SMPD3 cell lines produced less infectious EV. In the absence of nSMase2, cells produced more EV but there were fewer protected genomes associated with the EV. Knockdown of Alix or TSG101 had no effect on the infectivity of EV or the production of EV.

Summary/Conclusion: Overall, our studies found that biogenesis of JCPyV associated EVs depends upon the enzymatic activity of nSMase2 and not the ESCRT-related proteins Alix or TSG101.

Funding: NIH R01NS043097.

OWP2.08=PF05.09

Exosomes mediate the antiviral activity of interferon-β against Zika virus infection

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Introduction: IFNβ-induced exosomes (Exo-IFNβ) may impact on viral dissemination or antiviral immunity and therefore involve in the pathogenesis of many infectious pathogens. However, little is known about its underlying mechanisms. To better understand how Exo-IFNβ perform its antiviral effect, we employed

RNA sequencing analysis to explore the exosomal expression profiles of lncRNA and mRNA related to viral infections. We hypothesized that exosomes can regulate viral infection through transmitting enclosed specific lncRNAs into neighbouring cells to inhibit viral replication.

Methods: Exosomes were purified from A549 with/ without IFNβ treatment by serial centrifugation followed by sucrose density gradient purification, and characterized by TEM and Western Blot. ELISA assay were performed on purified exosome fractions to demonstrate that they are free of IFNβ. Zika virus (ZIKV) replication was assayed by real-time PCR.

Results: ZIKV replication was significantly suppressed in A549 cells pretreated with Exo-IFNβ followed by ZIKV infection. Moreover, we found that anti-ZIKV effect of Exo-IFNβ is IFN-independent because ZIKV replication was also decreased in U5A cells (IFN-α/β receptor IFNAR deficient) pre-treated with Exo-IFNβ. Similar results were observed in Dengue virus and HCV infections. RNA sequencing analysis found several lncRNAs and mRNAs were differentially expressed and function annotation and pathway analysis demonstrated that the differentially expressed genes were involved in many functions and pathways, including antiviral infection. To validate the RNA sequencing analysis results, some lncRNAs were selected to test their expression levels by qPCR. We are in the process of deciphering the mechanism employed by these exosomal lncRNAs in antiviral activity independent of inteferon.

Summary/Conclusion: We believe that understanding the antiviral functional molecules wrapped in exosomes may help design exosomes as efficient vehicles for antiviral therapy.

Funding: Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (2016-12M-3-025)

OWP2.09=PS02.09

Deciphering the role of extracellular vesicles on the blood–brain barrier during Zika virus infection

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Introduction: The association of Zika virus (ZIKV) with severe neurological disorders has gained increased interest over the last decade. However, the mechanism by which ZIKV crosses the blood–brain barrier (BBB) and reaches the brain remains to be elucidated. It is known that viruses incorporate viral material in extracellular vesicles (EVs) as a spreading strategy. These membrane-enclosed vesicles play a vital role in intercellular communication. Currently, there is a lack of knowledge on the possible involvement of EVs in ZIKV pathogenesis. Our study aims to unravel the role of EVs in ZIKV RNA transmission to the brain, via the BBB.

Methods: Human brain microvascular endothelial cells (HBMEC/D3) were used in our study since they represent the BBB *in vitro*. Three different EV isolation methods (precipitation kit, density gradient and size exclusion chromatography combined with the density gradient) were performed. Western blot, Transmission electron microscopy and Nanosight tracking analysis confirmed the presence of EVs in the supernatant of HBMEC/D3 cells. The presence of ZIKV RNA in infected-EVs (IEVs) was evaluated by immunofluorescence and qPCR. In addition, the effect of IEVs on the BBB was assessed using a label-free impedance-based biosensor (ECIS, Applied BioPhysics).

Results: We confirmed the presence of viral components in our IEVs, including the NS1 and E proteins of ZIKV. The obtained IEVs were able to reinfect susceptible cells, even after being pretreated with RNase A. This indicates that the viral RNA resides inside the IEVs. Using impedance measurements on HBMEC/ D3 cell monolayers, we observed that IEVs, as well as virus control caused similar and temporal disturbances on the monolayer's integrity within 30 min post infection. No disturbances were seen upon addition of non-infected EVs.

Summary/Conclusion: Our study demonstrates that EVs-derived from ZIKV-infected cells are able to transfer proteins and viral RNA to recipient cells. Since both IEVs and viral particles can induce similar changes on barrier's integrity it is possible that IEVs are involved in an alternative mechanism of ZIKV transmission.

OWP2.10=PF12.10

HIV-specific antibody mediated targeting of ENV+ tissues by exosomes

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Introduction: Antiretroviral therapy can effectively suppress HIV replication in the peripheral blood to an undetectable level. However, efforts to eradicate the latent virus in reservoirs remain a challenge and are a major obstacle in the treatment of HIV patients. Exosomes exhibit huge promise as an endogenous drug

delivery nanosystem for delivering drugs to reservoir tissues given their unique properties, including low immunogenicity, innate stability, high delivery efficiency and mostly importantly the ability to penetrate solid tissues due to their lipophilic properties.

Methods: In this study, we engineered and expressed the ScFv of a high affinity HIV-specific monoclonal antibody, 10E8, on exosome surface. Exosomes from 293T cells were loaded with curcumin via saponin, with efficient up to 34%. 10E8ScFv-expressing exosomes (10E8-Exo) showed highly efficient targeting of and curcumin delivery to CHO cell that expresses a trimeric gp140 on its surface (ENV+ cells) *in vitro* as demonstrated by confocal imaging and flow cytometry. We showed that 10E8-Exo could effectively bind to CHO cell that expresses a trimeric gp140 on its surface. The exosomes loaded with curcumin, a chemical that was shown to kill HIV-infected cells, showed specific killing of the trimeric gp140-expressing CHO cells. In an NCG mouse model that was grafted with the tumorigenic gp140-CHO cells and developed solid tissue tumours intravenously injected 10E8-Exo targeted the ENV-expressing tissues and delivered curcumin to induce a strong suppression of the ENV+ tumour growth with a low toxicity.

Results: Our results demonstrated that engineered exosomes can deliver anti-HIV agents to solid tissues by specifically targeting cells expressing viral env and induce cell killings.

Summary/Conclusion: It suggesting that such an approach can be developed for eradicating virus-infected cells in tissue reservoir.

Funding: This study was supported by The National Key Research and Development Program of China (2016YFC1201000), Nature Science Foundation of Jiangsu Province (BY2015069-02) and National Nature Science Foundation of China (81672020). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

OWP2.11=PS02.10

In vivo testing of OMV-based vaccine prototypes against _Gallibacterium anatis_

Fabio Antenucci[a], Homa Arak[b], Jianyang Gao[b], Toloe Allahghadry[b], Ida Thøfner[b] and Anders Miki Bojesen[c]

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Introduction: Outer membrane vesicles (OMVs) are produced by the majority of Gram-negative bacteria. Thanks to the antigenic similarity between OMVs and the bacterial outer membrane, OMVs have proven to be promising for the development of novel vaccines against bacterial pathogens. In this work, we describe the testing of OMV-based vaccine prototypes against *Gallibacterium anatis*, a Gram-negative pathogen of great veterinary interest.

Methods: OMVs were isolated from a *G. anatis* hypervesiculating mutant using a modified version of the Hydrostatic Filtration protocol described by Musante et al. (2014). 120 16-week-old Lohmann-Brown chickens were divided in six groups and immunized twice intramuscularly with different combinations of buffer (controls), OMVs and selected recombinant immunogens. Two weeks after second immunization, the effectiveness of the immunization regimes adopted was tested by challenging the animals intraperitoneally with live CFUs from a heterologous *G. anatis* strain. One week post-challenge, the animals were sacrificed and an established lesion score model was used during necropsy to evaluate the clinical outcome of infection.

Results: Statistical analysis of the recorded lesion scores showed that the group immunized with *G. anatis* OMVs presented an average total score of 2.95, as opposed to an average total score of 8.77 in the control group. The approximately three-fold reduction in total average lesion score observed demonstrates that immunization with *G. anatis* OMVs is able to effectively decrease the morbidity of *G. anatis* infection in the immunized animals.

Summary/Conclusion: Our results show that *G. anatis* OMVs represent a promising candidate for the development of cost-effective vaccination strategies for the prevention of *G. anatis* infections in a cross-serovar manner. Accordingly, we hypothesize that dose/response optimization and the enrichment of *G. anatis* OMVs with selected immunogens should result in an improvement of the effectiveness of the vaccination regime proposed.

Funding: This research project is being funded by a grant from Huvepharma (https://www.huvepharma.com/).

OWP2.12=PT05.04

Identification of a protein that presumably controls bacterial vesiculation in response to the extracellular environments

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Introduction: Many bacteria utilize extracellular membrane vesicles (EMVs) for survival in their growing

environments through communication with others, pathogenesis, and biofilm formation. Therefore, the amounts and the components of EMVs should be tuned in response to the conditions. Although several vesiculation mechanisms are suggested, little is known how bacteria control vesiculation in response to the environments. A bacterium *Shewanella* sp. HM13 has nine fold higher lipid-secretion capability in EMV fractions than *Escherichia coli*, and its EMVs contain a major protein (P49), which is not required for vesicle production. We used mutant EMVs that lack P49 to identify minor components of EMVs that may control vesiculation.

Methods: EMVs were subjected to 2D gel-based proteomics by peptide mass fingerprinting. Within the identified proteins, the function of a sensor protein homolog, HM1275, was analysed by swarming assay and lipid-staining to quantify EMVs produced in various media. Changes in the number of EMVs depending on culture media were quantified by tunable resistive pulse sensing method.

Results: A protein with a PAS domain and a methyl-accepting chemotaxis protein (MCP) sensing domain, HM1275, was identified in the EMVs. Although some MCPs are related to flagellar motility by binding some attractants, the flagellar motility of Delta-hm1275 was not significantly different from that of WT. Although the amounts of EMVs produced by WT were increased in response to the concentration of casamino acids in poor nutrient medium, those by Delta-hm1275 were not.

Summary/Conclusion: A putative sensor protein, HM1275, was identified in EMVs and may recognize the extracellular environments by binding signal molecules in casamino acids to control vesiculation. Although further studies are required to reveal the signals and the sensing pathways, the results obtained in this study indicate that bacterial vesiculation is controlled by extracellular environments, and artificial control of vesiculation with extracellular signals would be useful in applications such as suppression of vesicle-dependent pathogenicity.

Funding: Japan Society for Promotion of Science Research Fellowship for Young Scientists

OWP2.13=PT05.05

Prokaryotic BAR domain-like protein BdpA promotes outer membrane extensions

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Introduction: Bin/Amphiphysin/RVS (BAR) domains belong to a superfamily of membrane-associated coiled-coil proteins that influence membrane curvature. BAR domains are ubiquitous in eukaryotes and associated with membrane curvature formation, vesicle biogenesis/trafficking, protein scaffolding and intracellular signalling. While advances in protein domain prediction have facilitated the identification of several BAR domain proteins, they have yet to be characterized in bacteria. Here we identified a putative BAR domain-containing protein enriched in the outer membrane vesicles (OMVs) of *Shewanella oneidensis* MR-1, a dissimilatory metal-reducing bacteria known to produce outer membrane extensions (OMEs) that are suspected to facilitate long distance extracellular electron transfer (EET) but whose physiological relevance and mechanism of formation remain unknown.

Methods: Purified *S. oneidensis* OMVs were prepared by filtration and ultracentrifugation for comparative proteomics with cell-associated outer membrane proteins or for electrochemical measurements. Protein domains were predicted using HMMSCAN and CDD-search. OME formation and phenotype analyses were performed in situ by confocal and cryo-electron microscopy.

Results: The putative BAR domain-like protein BdpA was highly enriched in OMVs compared to cell-associated outer membranes. During OME biogenesis, WT *S. oneidensis* OMEs progress from elongated vesicle chains to narrow, tubule-like extensions while ΔbdpA OMEs remain as disordered vesicle chains. Purified OMVs from these strains are electrochemically active, with redox signals consistent with multiheme outer membrane cytochromes, supporting the role of OMEs in EET. Heterologous BdpA expression promotes OME formation in Marinobacter atlanticus and *Escherichia coli*, suggesting BdpA membrane sculpting activity is inducible and transferrable.

Summary/Conclusion: The ability of BdpA to promote OME formation and maturation into tubules *in vivo* supports BdpA as a comparator for BAR domain protein activity in bacteria.

Funding: US DoD Synthetic Biology for Military Environments (SBME) Applied Research for the Advancement of Science and Technology Priorities (ARAP)

NSF Dimensions: DEB-1542527

US DOE: DE-FG02-13ER16415

OWP2.14=PF07.10

Isolation of extracellular vesicles from extracellular matrix based hydrogel 3D cell cultures

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Introduction: Cancer-derived extracellular vesicles (EVs) are commonly studied and isolated from two-dimensional (2D) cell cultures. Nevertheless, three-dimensional (3D) culture systems with extracellular matrix (ECM) provide physiologically more relevant system to mimic *in vivo* tumour growth and progression of invasion. However, there are currently no methods to efficiently isolate EVs from ECM-based 3D cultures. For that purpose, we established a protocol for isolating EVs from cancer cells growing in a 3D ECM-based hydrogel.

Methods: Human prostate cancer PC3 cells were grown in 3D to form spheroids in a commercially available ECM-based hydrogel and the growth media was collected every two days for a period of 14 days, during which the spheroids grew invasive. The respective media were differentially centrifuged at 2, 10 and 100 K*g* and the pellets were resuspended in PBS. The EVs were analysed by western blotting (WB) against the common EV markers CD81, CD63 and CD9.

Results: Our preliminary data shows a step-wise increase of the EV markers in the media as the PC3 spheroids formed, expanded and invaded to the surrounding 3D ECM. The EVs produced by non-invasive or invasive spheroids are currently being characterized with nano tracking analysis, electron microscopy and WB.

Summary/Conclusion: This study demonstrates that EVs can be isolated from 3D ECM-based hydrogel cell cultures, which recapitulate the tissue architecture of solid tumours. Our results suggest that 3D cancer cell cultures have dynamic EV secretion determined by the phenotype of the spheroids. Taken together, we present a novel protocol for EV isolation from a 3D culture system and provide a platform to investigate EVs from *in vivo* mimicking conditions.

Funding: This project is funded by Magnus Ehrnrooth Foundation, K. Albin Johansson Foundation and Åbo Akademi University.

OWP2.15=PT07.07

Diagnostic microRNA biomarkers from circulating extracellular vesicles for early detection of pneumonia and severe secondary complications

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Introduction: Pneumonia remains one of the most deadly communicable diseases, causing three million deaths worldwide in 2016. Extracellular vesicles (EVs) are pivotal during signal transfer in the pathogenesis of inflammatory lung diseases. Since identifying pneumonia is particularly challenging in high risk groups (e.g. the elderly or infants), which often present with atypical symptoms and are at high risk for secondary complications such as sepsis or acute respiratory distress syndrom (ARDS), new approaches for early diagnosis are required. In this study we identified EV microRNAs (miRNAs) as potential biomarkers for inflammatory changes of the pulmonary tissue.

Methods: Our study included 13 patients with community-acquired pneumonia, 14 ARDS patients, 22 patients with sepsis and 31 healthy controls. After precipitating EVs from 1 mL serum, total RNA was extracted. Subsequent to library preparation and small RNA-Seq, differential gene expression analysis was performed using DESeq2. Data were filtered by mean miRNA expression of ≥50 reads, minimum twofold up or down regulation and adjusted p-value ≤0.05.

Results: The mean relative miRNA frequency varied slightly between the different groups and was highest in volunteers. Short sequences (<16 nucleotides), probably degradation products from longer coding and non-coding RNA species, were predominantly detected in patients. Based on unsupervised clustering, patients could be distinctly separated from healthy individuals. Although 21 miRNAs were significantly regulated in all patient groups compared to healthy controls, different disorders showed unique miRNA expression profiles. Distinct miRNA subsets were identified, which are

applicable to indicate disease progression from limited inflammation present in pneumonia to severe inflammatory changes as seen in ARDS and sepsis.

Summary/Conclusion: This study shows that EV miRNA biomarkers have potential for diagnosis of pneumonia and to indicate disease progression towards severe lung injury. Our findings are of clinical relevance, as the timely diagnosis of pneumonia can be challenging, and secondary complications such as ARDS and sepsis might be prevented by early intervention and treatment.

Funding: This study was supported by the German Federal Ministry for Economic Affairs and Energy under the programme "Zentrales Innovationsprogramm Mittelstand".

Oral with Poster Session 3
Chairs: Michael Pfaffl; Ryuichi Ono
Location: Level B1, Hall A

13:30–14:15

OWP3.01=LBT02.02

Using plasma to identify neural biomarker for antidepressant response in a treatment resistant cohort

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Introduction: Small extracellular vesicles (SEV) have emerged as candidate biomarkers in many complex diseases. An important characteristic of SEVs is their ability to bidirectionally cross the blood-brain barrier. This is particularly important in the context of major depressive disorder (MDD), where biomarkers are obtained from peripheral tissue and have been hard to relate to changes in brain functioning. 60% of MDD patients do not respond to their first antidepressant drug therapy (ADT) and treatment options are entirely at the discretion of the physician. Findings that can predict ADT response as well as provide insight into central mechanistic changes could revolutionize MDD treatment. The aim of this study is to profile exosomal microRNA (miRNA) in the context of ADT response in people with treatment-resistant depression. miRNA can act as biomarkers and might influence recipient cells to provide insight on disease-relevant mechanistic changes.
Methods: This pilot uses plasma from 10 controls and 10 patients with MDD (5 ADT responders (RES), and 5 non-responders (NRES)) from baseline (T0, before treatment). SEVs were isolated using a size exclusion column from Izon Science (Christchurch, New Zealand). Each isolation was divided into a "whole exosome" fraction and an immunoprecipitated "(NDE)" fraction using neural marker L1CAM. Quantitation and size determination was done using Tunable Resistive Pulse Sensing (TRPS) on the qNano gold. RNA was also extracted from SEVs from both fractions. The 4N-small RNA-Seq (Galas) protocol was used for library preparation.
Results: We found that the range of SEVs in the NDE fraction was smaller than the pool of all exosomes combined. Further, SEVs from all depressed patients were significantly smaller than controls irrespective of the fractions. Our sequencing results showed an increase of miR-151a-3p and miR-3168 in NRES, and miR-22-3p in RES. These results were specific to the NDE fraction.
Summary/conclusion: We have identified three potential biomarkers for ADT response which are uniquely present in the neural-derived fraction of peripheral SEVs.
Funding: Canadian Institutes of Health Research

OWP3.02=PT09.13

Immunocapturing of tumour-derived extracellular vesicles on micropatterned and antibody-conjugated surfaces for individual correlative light, probe and electron measurements

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Introduction: Tumor-derived extracellular vesicules (tdEVs) are promising biomarkers for cancer patient management. The screening of blood samples for tdEVs shows prognostic power comparable to screening of tumour cells. However, due to the overlap in size between tdEVs, non-cancer EVs, lipoproteins and cell debris, new approaches, not only based on size, are required for the reliable isolation of tdEVs and their quantification. We report an integrated analysis methodology to study single tdEVs using correlative data from scanning electron microscopy (SEM), Raman imaging and atomic force microscopy (AFM) to obtain a comprehensive dataset allowing identifying features unique to tdEVs.
Methods: Indium tin oxide (ITO)-coated fused silica was selected for its low Raman background. Substrates (1×1 cm^2) featuring position-dependent markings ("navigation marks") patterned by photolithography were modified with a monolayer of amino dodecyl phosphonic acid. The amine moieties were next reacted with poly(ethylene glycol) diglycidyl ether, forming an anti-biofouling layer. Anti-EpCAM antibodies were subsequently covalently bound on this surface. Samples of both tdEVs obtained from LNCaP cell lines and RBC-derived EVs were then introduced to

the surfaces. Finally, non-specifically bound EVs were washed away before SEM, AFM and Raman measurements were performed.

Results: Multiple objects were captured on the fully functionalized ITO surfaces, according to SEM imaging, while in negative control experiments (lacking functionalization or lacking antibody or using EpCAM-negative EVs), no object was detected. Principal component analysis of their Raman spectra, previously demonstrated to be able to distinguish tdEVs from RBC-derived EVs, revealed the presence of characteristic lipid bands (e.g. 2851 cm^{-1}) in the captured tdEVs. AFM showed a surface coverage of ~4×10^5 EVs per mm^2 with a size distribution similar to that found by NTA.

Summary/Conclusion: A platform was developed for multi-modal analysis of selectively isolated tdEVs for their multimodal analysis. In the future, the scope of this platform will be extended to other combinations of probe, light and electron microscopy techniques to relate additional parameters describing the captured EVs.

Funding: Funded by NWO Perspectief.

OWP3.03=PT09.14

The development of a scalable extracellular vesicle subset characterization pipeline

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Introduction: Liquid biopsies offer an important alternative to tumour biopsies that may be limited by the challenges of invasive procedures. We hypothesize that circulating Extracellular Vesicles (EVs) and their cargo may provide a useful surrogate biopsy method. Due to their small diameter (30–1000 nm), EVs migrate from the tissue into the peripheral circulation and provide a snapshot of the producing cells. Our lab has developed a first-in-class pipeline to use single cell – omics methods to characterize EV heterogeneity with high-sensitivity by combining multiplex assays and our custom MultiPlex Analysis post-acquisition analysis software (MPA_{PASS}), with subsequent high-resolution, single EV flow cytometric (FCM) methods.

Methods: A stan-dalone software package was developed in MATLAB to allow importation of multiplex flow cytometry output data. The package enables data quality screening of detection antibodies, bead recovery and data normalization methods. The software is equipped to handle large data sets comprising hundreds/thousands of phenotypes and samples. Data can be visualized in a variety of ways along with clustering using multidimensional data analysis techniques. All software outputs can be exported in a standardized templates containing metadata for reporting, as well as uploaded into atlases such as Genboree, where multiplex data can be stratified by RNAseq datasets. Analysis using this pipeline has been conducted using human samples from a variety of mediums including CSF, serum and plasma comparing EV phenotypes.

Results: Our multiplex approach and MPA_{PASS} software allows the use of single cell -omics tools for EV subset analysis in a manner that will elucidate the biological significance and function of different types of EVs. This high-throughput pipeline evaluates hundreds of EV protein profiles and will allow evaluation of millions of RNA:protein profiles in an unprecedented manner. Integration of RNA sequencing with protein characterization could provide an entirely new way of understanding EV regulation and function.

Summary/Conclusion: Our data show this form of EV profiling provides a way to monitor clinical responses early in the course of treatment, which may ultimately improve patient care and outcomes.

OWP3.04=PS04.13

An integrated microfluidic device for selective exosome isolation from human plasma

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Introduction: Extracellular vesicles released by many cell types circulate in blood vessel and play a key role in intercellular communication. Exosomes are 30–150 nm membrane vesicles and are also shed by both normal and cancer cells. Cancer cells are known as very heterogeneous, so exosomes are also heterogeneous and have different surface expression markers. Cancer-derived exosomes contain unique cargo determined by the molecular characteristics of cancer cells. Therefore, it is very important to selectively separate exosomes depending on surface expression for downstream analysis. We designed an integrated microfluidic chip for selective exosome isolation. The microfluidic chip consists of Hoof Structure (HS) for mixing exosomes and two different sized aptamer-coated particles and Multi-Orifice Flow Fractionation (MOFF) for separating each particle.

Methods: Biotinylated EpCAM aptamer was immobilized on the surface of 7 μm streptavidin-coated polystyrene particle and HER2 on 15 μm. The HS has the circular expansion channel on the 1st layer to generate expansion vortices and the two curvature channels on the 2nd layer to make chaotic advection. It makes transverse flow and mixes two particles without particle focusing phenomenon. The 100-nm (exosome), 7- and 15-μm fluorescence particles were used to test mixing performance between exosomes and particles in the HS. The MOFF was designed by a series of contraction/expansion microchannels for continuous size-based separation. Separation performance was tested by using the 7- and 15-μm fluorescence microparticles in the MOFF.
Results: The mixing efficiency was the highest at the flow rate 150 μL/min. Each exosome was continuously captured by aptamer-conjugated particle in the HS channel. The capture efficiency of EpCAM positive exosome was 96.9% and HER 2 was 68.09%. Two particles were separated in the integrated microfluidic device at the same flow rate. Also, 96.26% of 15-μm microparticles were positioned into the centre of the channel and 89.48% of 7 μm microparticles were separated on both sides of the channel.
Summary/Conclusion: Each exosome was continuously captured by mixing aptamer-conjugated particle in the HS. Exosome-conjugated microparticles were successfully separated by inertial force in MOFF. This analysis of each exosome will shed light on diagnosis and therapy of cancers.

OWP3.05= PF10.11

Aqueous two-phase system to isolate extracellular vesicles for prostate cancer diagnosis

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Introduction: Analysing extracellular vesicles (EVs) is an attractive means in prostate cancer diagnosis. However, existing methods of EVs isolation have low efficiency, purity and long process time, which induce low diagnostic ability. To approach the problems, we adapt a two-phase system to diagnose prostate cancer by isolating EVs from patients' urine. Using the two-phase system, prostate hyperplasia (BPH) patients and prostate cancer (PCA) patients were diagnosed, and the diagnostic ability was compared with conventional diagnostic methods.
Methods: Forty-two prostate cancer (PCA) patients and 20 benign prostate hyperplasia (BPH) patients' urine, plasma, saliva was collected and used for identifying EVs isolation ability of aqueous two-phase system (ATPS) and for comparing diagnostic ability of ATPS with conventional diagnosis.
Results: With an optimized ATPS, EVs were isolated with an efficiency of approximately 90%. In addition, the EV-isolation time was within approximately 30 min, and the purity of EVs in ATPS was approximately two times better than achieved with a conventional methods, ultracentrifugation and polymeric precipitation. After the ATPS isolated EVs from patients' body fluid, PCR and ELISA were utilized to detect EVs derived from prostate cancer cells. The expression levels of RNA and protein markers of prostate cancer were compared, and the relationship between expression levels and clinical data was analysed. The results demonstrated that diagnostic ability based on ATPS was better than other conventional methods (serum PSA and sediments). Moreover, sensitivity increased by at least 10%, and specificity was improved by at least 20% compared to conventional methods.
Summary/Conclusion: High quality and quantity of EVs can be obtained from patients' body fluid using ATPS. Using the abundant sources, which contains cancer-related protein and genes, we can perform a diagnosis with high specificity and sensitivity. Therefore, ATPS offers a powerful tool for more specific and sensitive diagnosis.

OWP3.06=PS05.11

***In vitro* and *in vivo* investigation of extracellular vesicles (EVs) as biomarker carriers in the diagnosis of early Alzheimer's disease**

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Introduction: Extracellular vesicles (EVs) represent an ideal source of biomarkers due to their role in cellular communication and their ability to carry protein aggregates. The most investigated EVs are exosomes, active entities secreted from cells and able to cross the blood brain barrier. Several neurodegeneration-involved molecules may undergo intercellular spreading through exosome release. In Alzheimer's disease (AD), before clinical signs appear, several proteins implicated in exo- and endocytic pathways are altered. In this

scenario, the identification of a correlation between variations in proteins carried by EVs and the progression of AD is the main aim of our project.

Methods: We performed exosome isolation and characterization from H4-SW glioma cells (a cell model featuring mutated β-amyloid overexpression), as well as in mouse- (triple-transgenic mouse model for familial AD) and human-plasma samples (Mild Cognitive Impairment (MCI) and AD subjects). In every case, a differential centrifugation protocol was applied and exosomes were then characterized using Nanoparticle Tracking Analysis with the NanoSight. We then explored exosome content, specifically Amyloid Precursor Protein (APP) and its proteolytic fragments, Microtubule Associated Protein Tau (tau), Progranulin (PGRN protein), Soluble Triggering Receptor Expressed on Myeloid Cells 2 (sTREM2) and α-synuclein (α-syn), using Western blot and ELISA. L1CAM and CD63 were evaluated to define the neural-derived exosomes amount in human samples.

All the samples were collected after ethical committee approval respecting Helsinki's declaration. Informed consents were provided by all the subjects.

Results: Our preliminary results show that APP, PGRN and sTREM2 are carried by H4- and human plasma-derived EVs. H4-SW cell-culture medium and 3Tg mouse plasma had a decrease in the EVs number release (≈1*10e8 EVs/mL) in comparison to control (≈7*10e8 EVs/mL). This decrease was not found in human plasma samples.

Summary/Conclusion: EVs purified from H4-glioma cellular AD model, 3xTg mouse-, MCI- and AD-plasma samples carry proteins relevant for neurodegenerative diseases (NDs). EVs release is reduced in cellular and animal AD-models.

Funding: Horizon 2020 Marie Sklodowska-Curie Innovative Training Networks – Blood Biomarker-based Diagnostic Tools for Early Stage Alzheimer's Disease.

OWP3.07=PF12.07

Shed microvesicles released from human primary and metastatic colorectal cancer cell lines contain key cancer progression proteins and RNA species

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Introduction: Extracellular vesicles (EVs) function in bidirectional cell–cell communication and contribute to the sustained growth, invasion and metastasis of cancer cells within the tumour microenvironment (TME). EVs comprise two main classes – exosomes and shed microvesicles (sMVs, also termed microparticles and ectosomes) – with distinct modes of biogenesis. Within each EV class, subtypes exist that can be distinguished by their distinct protein/ RNA signatures. Whilst much is known about exosome cargo content and functionality, sMVs are poorly understood.

Methods: Here, we compare protein/ RNA profiles and functionality of sMVs and exosomes secreted from human primary (SW480) and metastatic (SW620) colorectal cancer cell lines. Milligram amounts of EVs were purified from cell culture media using a combination of differential ultracentrifugation/ isopycnic iodixanol density centrifugation. Label-free quantitative mass spectrometry was performed to obtain protein profiles for SW480-derived and SW620-derived sMVs.

Results: We show that sMVs, unlike exosomes, are ALIX-, TSG101-, CD63- and CD9- and contain a different suite of key cancer progression modulators. Protein/ RNA signatures for SW480-derived sMVs and exosomes differ from each other and also from their SW620-derived counterparts. SW480-derived sMVs are enriched in ITGA/B, ANXA1, CLDN7, CD44 and EGFR/NOTCH signalling networks, while SW620-derived sMVs are enriched in PRKCA, MACC1, FGFR4 and MTOR/MARCKS signalling networks. Fibroblast invasion capabilities of SW480-derived and SW620-derived sMVs are comparable.

Summary/Conclusion: Furthermore, we report for the first time a comprehensive biochemical/ functional analysis of a hitherto undescribed subpopulation of sMVs. We anticipate our *in vitro* findings will be a starting point for more sophisticated studies aimed at elucidating the biochemical and functional properties of EV subtypes *in vivo*. The emerging roles of specific EV subtypes in the TME we believe will alter our view of cancer biology and might present new targets for therapeutic intervention.

Funding: Funding support from La Trobe University, Melbourne, Australia.

OWP3.08=PF12.08

Mass spectrometry analysis of small extracellular vesicles isolated from ovarian cancer ascites

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Introduction: High-grade serous carcinoma of the ovaries, fallopian tube and peritoneum (HGSC) is the deadliest

gynaecological malignancy with 5-year survival rate below 30%. HGSC is frequently accompanied by ascites, a pathological accumulation of fluid in the peritoneum, which can be exploited as a liquid biopsy containing not only cancer cells, but also the tumour microenvironment including extracellular vesicles (EVs). Tumour cells produce substantially more EVs than healthy cells, thus malignant ascites is the source of enriched pool of EVs of HGSC origin.

Methods: Ascitic fluids depleted of cells were fractioned using size-exclusion chromatography and two fractions – containing and not containing EVs – were further analysed. In parallel, small EVs were also isolated from ascitic fluids using differential ultracentrifugation followed by purification step in sucrose/D2O cushion. In total, 24 malignant ascites and 5 non-malignant ascites were used for EV isolation and further analysed using high-resolution hybrid mass spectrometer Orbitrap Fusion Lumos Tribrid. The subsequent data visualization and statistical analyses were performed using in-house-developed pipelines in KNIME environment.

Results: We identified 2441 proteins, in total, in the EVs from the ascites among which 21 were present in all 29 EV samples and not in non-vesicular fractions. Several of these proteins were specifically enriched in small EVs in malignant ascites in comparison with non-malignant ascites. These proteins are now being evaluated as biomarkers.

Summary/Conclusion: Using advanced mass spectrometry, we identified candidate proteins which are specifically enriched in small EVs of HGSC. These proteins warrant further investigation as they may act as important players in HGSC progression as well as serve as potential prognostic/diagnostic/screening biomarkers of HGSC.

Funding: Czech Science Foundation, Grant No. GJ17-11776Y.

OWP3.09=PT09.12

Identification of single tumour-derived extracellular vesicles by means of optical tweezers and Raman spectroscopy

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Introduction: EVs derived from cancer cells play a role in tumour cell proliferation, migration, invasion and metastasis. Their presence in body fluids, such as blood, makes them potential biomarkers for cancer disease. However, the identification of single tdEVs can be challenging due to their heterogeneity, their ultra-small size, their size overlap with many other normal EVs and contaminants in body fluids and the lack of knowledge on their chemical composition.

Methods: Synchronized optical tweezers and Raman spectroscopy have enabled a study of individual EVs. The new method detects individual trapping events from Rayleigh scattering. The synchronous recording of Raman scattering enabled the acquisition of Raman spectra of both individual and multiple EVs, disclosing their chemical composition. Furthermore, Mie light scattering theory has been used to relate the Rayleigh scattering intensity to the size of trapped EVs.

Results: The light scattered of trapped EVs gave rise to step-wise time traces that can be used to distinguish individual trapping events from accumulative cluster events due to the discrete nature of the steps which correspond to single trapping events. Next, we confirmed the trapping of individual EVs derived from PC3 cells, red blood cells, platelets and blood plasma by acquiring both, Rayleigh and Raman scattering signals. While the step-wise trend in the Rayleigh scattering signal suggests trapping of single particles, the Raman scattering signal demonstrates the nature of the trapped EVs. Through principal component analysis (PCA), the main spectral variations among the four EV types were identified. The principal component scores grouped the PC3-derived EVs in a separate cluster from the rest of the EVs.

Summary/conclusion: We have developed an automated single particle optical tweezers – Raman and Rayleigh scattering setup to trap and release single EVs over time. We demonstrated single-EV trapping by simultaneous acquisition of Rayleigh and Raman scattering. PCA enabled the identification of single-EVs derived from the cancer cell line PC3. This discloses chemical information as a step towards the identification and characterization of single tumour-derived EVs in blood.

Funding: Cancer ID – project number 14193, (partially) financed by the Netherlands Organisation for Scientific Research (NWO)

Symposium Session 5: EVs in Infectious Diseases
Chairs: Shilpa Buch; Vera Tang
Location: Level B1, Hall A

14:15–15:00

OT05.01

Extracellular vesicles provide a capsid-free vector for oncolytic adenoviral DNA delivery

Heikki Saari[a], Tiia Turunen[b], Mikko Turunen[b], Matti Jalasvuori[c], Sarah Butcher[d], Seppo Ylä-Herttuala[b], Tapani Viitala[a], Vincenzo Cerullo[a], Pia Siljander[e] and Marjo Yliperttula[a]

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Introduction: Extracellular vesicles (EVs) have been showcased as auspicious candidates for delivering therapeutic cargo, including oncolytic viruses for cancer treatment. Delivery of oncolytic viruses in EVs could provide considerable advantages, hiding the viruses from the immune system and providing alternative entry pathways into cancer cells. Here we describe the secretion and viral cargo of EVs secreted by cancer cells infected with an oncolytic adenovirus (IEVs, infected cell-derived EVs) as a function of time after infection.
Methods: IEV-containing cell culture medium was collected from A549 and PC-3 cancer cell cultures every 24 h after being infected with an oncolytic adenovirus and IEVs were isolated by iodixanol density gradient centrifugation. IEVs were then characterized by cryo-TEM, NTA, immunoblotting and qPCR for structural properties and viral components and their infectivity was confirmed by cytotoxicity assay and TEM of IEV-treated cells.
Results: IEVs were secreted already before the lytic release of virions and their structure resembled normally secreted EVs, suggesting that they were not just apoptotic fragments of infected cells. IEVs were able to carry the viral genome and induce infection in other cancer cells. The amount of viral cargo associated with IEVs increased as the infection progressed, although no intact virions were observed in any of the IEVs visualized by cryo-TEM. The amount of viral cargo also appeared to be density-dependent, in that heavier IEVs contained more viral DNA and protein per vesicle.
Summary/Conclusion: Given that adenovirus is a DNA-virus that is assembled in the nucleus and released via induced lytic cell death, the generation of infective EVs as prescribed here suggests that part of the produced viral DNA is secreted via IEVs before cell lysis. As such, the role of EVs in the life cycle of adenoviruses may be an important part of the successful infection and may also be harnessed for cancer- and gene-therapy as vectors of viral DNA invisible to the immune system.

OT05.02

Bacterial growth stage regulates the size, composition and biological functions of membrane vesicles

Lauren Zavan[a], Natalie Bitto[a], Ella Johnston[a], David Greening[b] and Maria Kaparakis-Liaskos[a]

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Introduction: Outer membrane vesicles (OMVs) are naturally released by all Gram-negative bacteria as part of their normal growth and contain many of the components found in their parent bacterium, including DNA, RNA and proteins. To date, few studies have compared the proteome of OMVs to that of their parent bacterium and examined how it changes throughout bacterial growth. In this study, we aimed to elucidate the contribution of bacterial growth stage on the size, composition and biological functions of *Helicobacter pylori* OMVs.
Methods: OMVs were purified from *H. pylori* cultures grown to early log, mid log or stationary phase of bacterial growth, and their size and protein composition were analysed using NTA and proteomics, respectively. The ability of OMVs isolated from various growth stages to stimulate an inflammatory response in human epithelial cells was determined by ELISA.
Results: We found that OMVs became less heterogeneous in size throughout bacterial growth. We showed that the proteome of OMVs was vastly different to that

of their parent bacterium from each time point, suggesting that there is preferential cargo packaging of bacterial proteins into OMVs. Gene ontology and enrichment analyses identified that bacterial growth stage regulated the type of proteins packaged into OMVs, as early log and stationary phase OMVs were enriched in proteins required for metabolic pathways, whereas late log phase OMVs contained proteins contributing to cell signalling. Finally, we identified that bacterial growth stage affected the inflammatory response mediated by OMVs in host epithelial cells, highlighting that bacterial growth stage regulates the subsequent biological functions of OMVs.

Summary/Conclusion: Our findings identify that bacterial growth stage regulates the size, protein cargo composition and biological functions of *H. pylori* OMVs, and that therefore OMVs from various growth stages are not comparable. Collectively, these findings emphasise the importance of considering bacterial growth stage from which OMVs are isolated from, as this will ultimately affect their protein content and biological functions. We are currently determining whether bacterial growth stage also regulates the composition and functions of Gram-positive bacterial membrane vesicles.

Funding: Australian Research Council.

OT05.03

Can exosomes be used to predict where patients are on the tuberculosis disease spectrum?

Nicole Kruh-Garcia, Gustavo Diaz, Cristian Oliva Aviles and Karen Dobos

Colorado State University, Fort Collins, USA

Introduction: *Mycobacterium tuberculosis* (*Mtb*), the causative agent of the disease tuberculosis (TB), is a highly successful human pathogen. *Mtb* has the ability to survive within the host macrophage. In response to the challenges of the intracellular environment, the bacteria secretes a dynamic subset of proteins reflective of its metabolic state, some of which are entrapped in exosomes and released from the host cell. Our ultimate goal is to improve upon the current diagnostics to facilitate early and rapid diagnosis of active disease, which is a key to timely drug invention and further spread of the disease. Using serum exosomes, we aimed to determine if a proteomic fingerprint can be used to discriminate between individuals with TB from non-TB, as well as to classify TB suspects – individuals with pulmonary symptoms but without detectible mycobacteria in their sputum.

Methods: Hyper Reaction Monitoring Mass Spectrometry (HRM-MS) was applied to a sample set of serum exosomes isolated from four groups: TB negative (healthy non-endemic and TB-suspect endemic) and TB positive (smear negative or positive, all culture positive) individuals. This allowed us to decipher a host protein profile that is common with exosomes from individuals who have sputum confirmed TB and distinct from those of whom do not. Peptide intensities were normalized and differences were in abundance, which were determined by *t*-test.

Results: Nine proteins – including FCGR3A and α-2-HS-glycoprotein, show distinct patterns, either increasing or decreasing with disease severity. Using adaptive least absolute shrinkage and selection operator (Lasso) we are able to discriminate TB patients from healthy and TB suspects using nine proteins. Application of the model to a test set of smear and culture negative TB suspect serum exosomes, we found that nine were consistent with the negative diagnosis, while one surpassed the threshold for positivity. When the same sample was screened for the presence of mycobacterial peptides using our published targeted MS assays, it was positive for several Mtb peptides.

Summary/Conclusion: These results indicate that a novel assay detecting a combination of host and Mtb proteins can discriminate TB positivity with greater sensitivity than the current sputum diagnostics.

Funding: Bill and Melinda Gates Foundation Fund #: OPP1039688.

Symposium Session 6: EV Engineering I
Chairs: Hang Hubert Yin; Siyang Zheng
Location: Level 3, Hall B

13:30–15:00

OT06.01

Designed EVs for intracellular delivery of therapeutic antibodies

Oscar Wiklander[a], Dhanu Gupta[a], Joel Nordin[a], Heena Sharma[b], Xiuming Liang[a], Giulia Corso[a], Dara Mohammad[a], Rim Jawad[a], André Görgens[c] and Samir El Andaloussi[d]

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Introduction: Extracellular vesicles (EV) can be engineered to display various targeting and therapeutic moieties. Their ability to also act as a natural vector to shuttle cargo over biological barriers offers a unique platform for the development of a new class of therapeutics. Here, we introduce a novel concept consisting of antibody coupled therapeutic EV in order to target tissues or intracellular pathways.

Methods: By engineering EV to express an Fc-binding moiety (Fc-EV), antibodies can be displayed on the surface of the vesicles. We have extensively evaluated the capacity of these EV to bind antibodies by immuno-electron microscopy, cellular uptake of labelled antibodies/EV and flow cytometry analysis, which indicates that EVs can be decorated with antibodies. As a proof of concept, antibodies bound to Fc-EV, were assessed in inflammatory models as well as in cancer settings.

Results: Delivery of anti-STAT3 antibodies in an *in vitro* STAT3 dependent inflammatory reporter model was assessed, with promising results showing inhibition of STAT3 transcriptional activity. Furthermore, intracellular delivery of anti-STAT3 antibody using Fc-EV displays a dose dependent growth inhibition in pancreatic ductal adenocarcinoma (PDAC) cells. The Fc-EV platform can also be utilized for decorating EVs with cancer targeting antibodies, a feature that can be harnessed to address the differences in uptake displayed by different cancers. Certain cancer types are known to rapidly internalize EV, whereas other cancer types, such as malignant melanoma are known to take up EV to a very low extent, if taken up at all. Our results show that antibodies targeting surface molecules of cancer cells also aid the internalization of EV into cancer cells, thus further indicating the potential of utilizing EV as therapeutic vectors. In order to achieve specific targeting to B16F10 malignant melanoma cells, we have decorated the EV surface with antibody targeting surface proteins that are known to be displayed on B16F10 cells, which lead to cellular association of EV to these cells.

Summary/Conclusion: Overall the Fc-EV platform offers the prospective of combining antibody and EV technology, with potential applications including tissue and cell targeting as well as intracellular delivery of functional antibodies.

OT06.02

Extracellular vesicles derived from AT-MSCs mediated miR-424 delivery promote apoptosis via the PD-L1/PD-1 pathway in TNBC

Yueyuan Zhou[a], Nobuyoshi Kosaka[b], Zhongdang Xiao[c] and Takahiro Ochiya[b]

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Introduction: Extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) showed great potential as the delivery vehicle of drugs including miRNAs based on its low immunogenicity and natural homing ability. Triple-negative breast cancer (TNBC) is an aggressive and invasive subtype that has limited treatment options. Meanwhile, TNBC is immunogenic with a greater percentage of tumour-infiltrating lymphocytes and increased expression of the programmed death-ligand 1 (PD-L1) in the tumour microenvironment. The aim of our study is to apply MSC-EVs to modulate the expression of PD-L1 via the delivery of miR-424 and contribute to the immunotherapy for TNBC.

Methods: EVs generated from adipose tissue-derived MSCs (AT-MSCs) were isolated by differential centrifugation and characterized by western blot, nanoparticle tracking analysis and transmission electron microscopy. Before EV collection, AT-MSCs were modified to overexpress miR-424 through electroporation, and miRNA mimics transfection. The miRNAs targeting PD-L1 was predicted according to in silico analysis. The direct regulation of miR-424 on PD-L1

was verified via the 3'-UTR luciferase report assays. The purified EVs were added to the recipient MDA-MB-231 cells (MM-231). The expression of PD-L1 mRNA and protein was analysed via qRT-PCR and western blot, respectively.

Results: We found that miR-424 directly regulated the expression of PD-L1 through the binding to PD-L1 3'UTR. Furthermore, the expression of PD-L1 in MM-231 cells was down-regulated and the expression of miR-424 in MM-231 was up-regulated after coculture with exosomes derived from normal AT-MSCs, and AT-MSCs with miR-424 overexpression. Moreover, the cell viabilities of MM-231 were decreased after coculture with exosomes or transfected with miR-424 mimics.

Summary/Conclusion: EVs derived from AT-MSCs could transfer functional miR-424 to TNBC cell lines and promote the apoptosis via decreased immune-negative PD-L1/PD-1 pathway.

Funding: This work was supported by Project for Cancer Research and Therapeutic Evolution [P-CREATE; grant number:17cm0106402h0002], MEXT KAKENHI [Grant-in-Aid for Young Scientists (A); grant number: 17H04991] and China Scholarship Council [grant number: 201706090122].

OT06.03

Exosomal delivery of NF-κB repressor delays LPS-induced preterm birth in mouse models

Samantha Sheller-Miller[a], Kyungsun Choi, George Saade, Chulhee Choi[b] and Ramkumar Menon

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Introduction: Intraamniotic infection and inflammation are associated with spontaneous preterm birth (PTB) and preterm premature rupture of the membranes (pPROM). In this study, we tested engineered extracellular vesicles, or exosomes, cargoing an inhibitor to pro-inflammatory transcription factor (NF-kB), called super-repressor (SR) IkB, to prolong gestation in an infection (LPS)-induced PTB mouse model.

Methods: HEK293T (human embryonic kidney cell) derived exosomes were engineered to contain SR using a protein loading via optically reversible protein–protein interaction (EXPLORs) method (Yim, et al 2016). In this method, SR is actively incorporated into exosomes during biogenesis. These exosomes were isolated, quantified and used for our studies. Intraperitoneal (IP) injection of either LPS (100 g) or PBS were performed in CD-1 mice on gestational day 15 followed by injection of PBS, SR exosomes (1 × 1010) or naïve exosomes (exosomes derived from HEK293T cells under normal culture conditions, 1 × 1010) every 2 h for a total of five injections. Treatment groups (Group 1-LPS+PBS; Group 2-LPS +SR; Group 3-LPS+naïve, and Group 4-PBS) were monitored for preterm birth. Upon delivery of at least one pup in Group 1, mice were euthanized, and maternal plasma, uterus and cervix were collected for cytokine analysis using Luminex (IL-1β, IL-8 and IL-10) and Western blot for NF-B activation via RelA phosphorylation (P-NF-B), respectively. Survival graphs were created in GraphPad and one-way ANOVA was performed to determine statistical significance ($P < 0.05$).

Results: Animals injected with PBS delivered at the expected gestational age (19.5 days). LPS and LPS + naïve-induced PTB within 10 h; however, injection of SR exosomes prolonged delivery by an average of 21 h in this model. Consistently lower levels of pro-inflammatory cytokines, IL-1β and IL-8, were seen in maternal plasma of LPS + SR compared to LPS mice, while anti-inflammatory cytokine, IL-10, levels were significantly increased in LPS + SR mice compared to LPS ($P = 0.01$) and PBS controls ($P < 0.0001$). In the cervix and uterus, P-NF-B expression was significantly decreased in LPS + SR compared to LPS ($P = 0.005$, $P = 0.03$) (Figure 2B).

Summary/Conclusion: Exosomes can be engineered to carry pharmaceutical agents that can dampen the infection-induced inflammation associated with PTB and pPROM.

OT06.04

Technologies for loading RNA-based therapeutics into extracellular vesicles for drug delivery

Olga Shatnyeva[a], Anders Gunnarsson[b], Euan Gordon[c], Elisa Lázaro-Ibáñez[d], Lavaniya Kunalingam[c], Xabier Osteikoetxea[e], Kristina Friis[c], Marcello Maresca[c] and Niek Dekker[b]

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Introduction: Extracellular vesicles (EVs) have emerged as a very potent new delivery system for drug delivery. Recent advances in RNA-based therapeutics have broadened the scope of cellular targeting of currently undruggable genes. Current approaches for RNA loading of EVs suffer from poor efficacy. Our study combines bioengineering of the therapeutic EVs with post-isolation RNA. We will here present data showing (1) the use of RNA binding proteins (RBP) fused to EV protein markers for *in vitro* loading of EVs with tagged RNA cargo and (2) post-isolation

incubation of EVs with RNA-loaded lipid nanoparticles (LNP).
Methods: A library of targeted RNAs fused to a specific RNA binding protein (RBP) sequence was generated, varying the position of recognition site. Surface plasmon resonance was used to characterize the modified sgRNAs for binding to the RBP. Activity of the hybrid sgRNA was also confirmed for functional gene editing with Cas9. Expi293F cells were co-transfected with the set of modified sgRNAs and RBP fused to EV proteins followed by EV purification by differential ultracentrifugation. EVs were characterized by nanoparticle tracking analysis, Western blotting and single molecule microscopy. Efficiency of sgRNA loading into EVs was determined using qPCR. Post-isolation loading of sgRNA with Expi293 EVs by co-incubation and functional delivery of sgRNA cargo in HEK293 cells were also evaluated.
Results: The introduction of RNA recognition elements into sgRNA sequence did not interfere with binding to RBP. Fusions between RBP and EV proteins resulted into efficient incorporation of RBP in EVs. Co-expression of sgRNA resulted in selective targeting of sgRNA to EVs. Additionally, EVs from cells co-expressing sgRNA and RBP contained 10-fold more sgRNA compared to EV from cells who only expressed sgRNA. Loading of synthetic sgRNA cargo with 40% encapsulation efficiency was achieved by incubation of EVs with LNPs and the resulting particles led to functional uptake in HepG2 cells.
Summary/Conclusion: Here, we compare different techniques for therapeutic cargo loading and delivery into target cells. All approaches for RNA loading into EVs demonstrates proof of principle. We envision that this approach will be useful for RNA loading for therapeutic applications.

OT06.05

Engineering designer exosomes produced efficiently by mammalian cells *in situ* and their application for the therapy of Parkinson's disease

Ryosuke Kojima[a], Daniel Bojar[b] and Martin Fussenegger[c]

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Introduction: Exosomes are cell-derived extracellular nanovesicles 50–150 nm in size, which serve as intercellular information transmitters in various biological contexts, and are candidate therapeutic agents as a new class of drug delivery vesicles. However, inefficiency of exosome cargo transfer, such as transfer of mRNA contained in exosomes, and lack of methods to create designer exosomes has hampered the development of sophisticated therapeutic interventions.
Methods: We have developed a set of synthetic-biology-inspired genetic devices that enable efficient customizable *in situ*-production of designer exosomes in engineered mammalian cells, and pursued their therapeutic applications.
Results: The developed synthetic devices that can be genetically encoded in exosome producer cells (named "EXOtic (EXOsomal Transfer Into Cells) devices") enhance exosome production, specific mRNA packaging and delivery of the mRNA into the cytosol of recipient cells. Synergistic use of these devices with a targeting moiety significantly enhanced functional mRNA delivery into recipient cells, enabling efficient cell-to-cell communication without the need to concentrate exosomes. Further, the engineered exosome producer cells implanted in living mice could consistently deliver mRNA to the brain. Moreover, therapeutic catalase mRNA delivery by designer exosomes attenuated neurotoxicity and neuroinflammation in both an *in vitro* and *in vivo* Parkinson's disease model.
Summary/Conclusion: These results indicate the potential usefulness of the EXOtic devices for RNA delivery-based therapeutic applications. (Nat. Commun. 2018, 9, 1305)
Funding: This work was supported by the European Research Council (ERC) advanced grant [ProNet, no. 321381] and in part by the National Centre of Competence in Research (NCCR) for Molecular Systems Engineering (to M.F.). R.K. was supported by a postdoctoral fellowship from the Human Frontier Science Program.

OT06.06

Protein engineering for loading of Extracellular Vesicles

Xabier Osteikoetxea[a], Josia Stein[a], Elisa Lázaro-Ibáñez[b], Gwen O´Driscoll[c], Olga Shatnyeva[d], Rick Davies[a] and Niek Dekker[c]

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Introduction: To date various reports have shown the utility of extracellular vesicles (EVs) for delivery of therapeutic protein cargo. Currently, the most common strategies for loading therapeutic cargoes occur after EV isolation mixing EVs with desired cargo and subjecting to passive incubation, electroporation, freeze-thaw cycling, sonication, extrusion, or membrane permeabilization with saponin among various

techniques. An alternative approach is to modify releasing cells to secrete EVs containing the desired cargo with minimal impact on native EVs by post-isolation treatments. In this study, we designed different constructs to compare Cre and Cas9 loading efficiency into EVs using (1) light-induced dimerization systems (Cryptochrome 2 (CRY2), Phytochrome B (PHYB), and Vivid-based "Magnets" (Mag)), (2) fusion to EV associated proteins (CD9, CD63, CD81, Rab5), (3) lipidation motifs (Myristoylation, Palmitoylation, Prenylation) and (4) a novel motif "mXO".

Methods: For EV production human Expi293F cell line was transiently transfected with plasmid constructs using PEI MAX 40K. EVs were then isolated by differential ultracentrifugation followed by iodixanol gradient and characterized using nanoparticle tracking analysis, western blotting and transmission electron microscopy. Functionality of engineered Cre and Cas9 cargo was assessed in reporter cell assays using fluorescent microscopy, qPCR and Sanger sequencing followed by TIDE analysis.

Results: Light-induced dimerization using CRY2 resulted in better EV cargo loading for both Cre and Cas9 than PHYB and Mag systems. Among the EV associated proteins and lipidation motifs tested CD9, CD81, Myristoylation and mXO were most efficient at recruiting Cre and Cas9 cargo by light-induced dimerization. Using CRY2 in combination with CD9 or mXO we achieved loading efficiencies of 10–34 Cre molecules per EV and 23–30 Cas9 molecules per EV.

Summary/Conclusion: Cre and Cas9 loading into EVs by producing cells is feasible using protein engineering with light-induced dimerization. Of the designs investigated CRY2-induced light dimerization and CD9 and mXO motif were most effective resulting in multiple copies of functional Cre and Cas9 loaded per EV. We envision that this approach will be useful for protein loading in a variety of therapeutic applications.

OT06.07

Engineered extracellular vesicles for drug delivery

Niek Dekker[a], Elisa Lázaro-Ibáñez[b], Olga Shatnyeva[c], Nikki Heath[d] and Xabier Osteikoetxea[e]

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Abstract: Extracellular Vesicles (EVs) represent an exciting opportunity as biological delivery vehicle for therapeutic cargo with excellent safety, low intrinsic immunogenicity, cell-specific tropism and biological delivery efficiency.

Methods: There are multiple approaches for the introduction of protein and RNA cargo into EVs, including physical, chemical and cell engineering. We have engineered Expi293F suspension cells with transient expression of fusion proteins for reversible loading with protein cargo with examples for Cre recombinase and Cas9 for CRISPR gene editing.

Results: We have developed a single molecule fluorescence microscopy technique to quantify cargo loading at a single particle level, showing excellent loading for GFP fusions with CD63 with on average 70 copies of the fusion protein per particle. Functional delivery of Cre recombinase, as measured in a reporter cell line, was dependent on addition of small molecule or peptide enhancers of endosomal escape. Using RNA-binding proteins fused to exosomal markers we were able to enrich EVs with RNA cargo with up to 10-fold higher loading of sgRNA compared to loading from passive mass redistribution. Human Expi293F cell-derived EVs did not trigger any significant immune response *in vitro* in human blood. The *in vivo* assessment following a single intravenous administration of these EVs in BALB/c mice did not reveal marked haematological changes, cytokine induction or histopathological effects. Labelling of EVs using fluorescent mCherry, luminescent NanoLuc or radio-isotope 111Indium marker allowed for on line analysis of bio-distribution *in vivo*.

Summary/conclusion: Opportunities of naïve and engineered EVs for drug discovery and their potential for therapeutic applications will be discussed.

PT01: Cellular and Organ Targeting
Thursday Poster Session
Chairs: Charles Lai; Ikuhiko Nakase
Location: Level 3, Hall A

15:30–16:30

PT01.01

Role of circulating extracellular vesicles in brain function and behaviour

Eisuke Dohi, Indigo Rose, Takashi Imai, Rei Mitani, Eric Choi, Dillon Muth, Zhaohao Liao, Kenneth Witwer and Shinichi Kano

Johns Hopkins University School of Medicine, Baltimore, USA

Introduction: Accumulating evidence suggests that extracellular vesicles (EVs) circulate in the blood and affect cellular functions in an organ distant from their origins. In neuroscience, systemic circulating factors such as cytokines/chemokines, hormones and metabolites have been shown to modulate brain function and behaviour. They are also utilized as biomarkers to reflect brain disease status. Nonetheless, it remains unclear whether circulating EVs modulate brain function and behaviour.

Methods: We used mouse models to study the effects of EVs from specific cell types on brain function and behaviour. Because circulating EVs are extremely heterogeneous, we focused on immunodeficient mice that lack specific lymphocytes (T and B cells). We assessed the changes in their circulating EVs and examined their potential impact on the corresponding behavioural and neuronal dysregulation.

Results: As expected, immunodeficient mice lack the expression of T and B cell-related markers in the EV containing fractions from the peripheral blood. Immunodeficient mice also displayed social behavioural deficits, accompanying by enhance c-Fos immunoreactivity in the excitatory neurons in the medial prefrontal cortex (mPFC). Notably, transfer of splenocytes from wild-type (WT) rescued the behavioural deficits, serum EVs and brain c-Fos expression patterns in immunodeficient mice. Further analysis on the molecular mechanisms is in progress.

Summary/Conclusion: Our study has revealed a potential periphery-brain communication via EVs under physiological condition. Future studies are required to identify the cellular targets of circulating EVs and their ascending routes in the brain.

Funding: NIMH R01.

PT01.03

In vivo tracking and monitoring of extracellular vesicles with a new non-lipophilic dye

Sam Noppen[a], Gareth R Willis[b], Antonios Fikatas[a], Archana Gupta[c], Amirali Afshari[c], Christophe Pannecouque[a] and Dominique Schols[a]

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Introduction: Extracellular vesicles (EVs) are gaining increasing interest as drug delivery vehicles. However, there is still a lack of knowledge about the *in vivo* fate of exogenous delivered EVs. Noninvasive optical imaging is an important tool to analyse the biodistribution of EVs. Currently, one of the most popular techniques is to directly label EVs with fluorescent lipophilic dyes. A major drawback is that the dye itself rather than EVs is detected. Hence, there is a need for other dyes that overcome these limitations. A new non-lipophilic near infrared (NIR) dye, ExoGlow-Vivo (SBI), was tested *in vivo* in mice.

Methods: EVs from human PBMC, HEK and MCF7 cells were labelled with ExoGlow-Vivo, precipitated with Exoquick-TC (SBI) and injected intravenously (i.v.) in adult SCID mice. Human mesenchymal stem cell (MSC)-derived EVs were labelled with ExoGlow-Vivo dye, washed via ultracentrifugation and injected i.v. in post-natal day-4 FVB mice. Fluorescent images were acquired with an IVIS® Spectrum (PerkinElmer).

Results: Spectral unmixing of ExoGlow-Vivo revealed optimal excitation/emission at 745/820 nm. Biodistribution studies in SCID mice showed the liver as main targeted organ. A high fluorescent signal was measured in the bladder but almost completely disappeared within 4 h. *Ex vivo* imaging of dissected organs confirmed the liver as the main organ, followed by spleen and kidneys. No difference in biodistribution was observed between PBMC, HEK and MCF7-derived EVs. MSC-derived EVs accumulated preferentially in the liver of post-natal mice. Interestingly, ex vivo imaging revealed high positive staining in the lungs. This may be associated with recent observations that found

MSC-derived EVs ameliorate core features of experimental bronchopulmonary dysplasia.

Summary/Conclusion: ExoGlow-Vivo has excellent NIR properties for *in vivo* imaging with almost negligible background. This non-lipophilic dye is ideal for biodistribution and kinetic studies of exogenous delivered EVs. However, uptake of most EVs by liver supplants the signal of other organs.

PT01.04

Exosomal lipids applicable to cancer targeting

Yuki Toda[a], Saeka Ukai[a], Akari Kobayashi[a], Shin-ya Morita[b], Shigekuni Hosogi[a] and Eishi Ashihara[a]

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Introduction: Cancer-targeting technologies must be a crucial technology to develop diagnosis and therapy of cancers. Many basic and clinical studies highlighted lots of cancer-associated proteins and expected their ligands to honour the drug carrier with selectivity; however, their targeting potential were not tolerable of advanced trials. We have previously identified glioblastoma-derived exosomes (Exo-U251) that were more effectively internalized into some types of cancer cell lines than into non-cancer cells. Because this tropism was still maintained in the lack of protein ligand interaction of the exosomes with cells, we focused on their lipid components.

Methods: Ultrapure exosomes (about 100 nm vesicles expressing CD63 in d = 1.16 mg/L fraction) were collected from cell culture media using density-gradient ultracentrifugation. Exosomal lipids were extracted by Bligh & Dyer method and reconstructed into liposomes (Exolip-U251) using extrusion method. Each amount of lipid components was analysed by enzymatic fluorometric assay [Morita S.Y. and Terada T. Sci. Rep., 5:11737, 2015]. Exosomes or reconstructed liposomes were fluorescent-labelled and applied to cancer or non-cancer cells to evaluate the internalization efficiency using an image analysis of a laser scanning microscopy. Exolip-U251 conjugating siRNA was prepared by Exo-Fect reagent. Doxorubicin (DOX) was encapsulated into liposomes using remote-loading method.

Results: The enzymatic fluorometric assays revealed the uniqueness of the exosomal lipid components according to the cells from which they are derived. The tropism of Exo-U251 lipid-reconstructed liposomes (Exolip-U251) partly mimicked that of the original exosomes. The siRNA conjugated Exolip-U251 was effectively delivered into the inside of cells, but did not suppress the target gene expression. By contrast, DOX-loaded Exolip-U251 significantly suppressed the proliferation of U251 cells. Thus, the encapsulation was critical for the agents to internalize more effectively into cancer cells.

Summary/Conclusion: The tropism of exosomes is partially regulated by their own lipid components and, mimicking this approach would lead to promising cancer-targeting technologies.

Funding: MEXT-Supported Program for the Strategic Research Foundation at Private Universities

PT01.05

The exosome that are released from mechanical stress-stimulated osteocyte induces osteoclastegenesis

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Introduction: Osteocyte, which is the most abundant cell in bone tissues, is well known as a mechanical stress receiving cell. During bone remodelling, bone resorptionby osteoclasts precedes bone formation by osteoblasts. However, its mechanism is still unknown. In this study, we examined whether exosome released from osteocyte by MS stimulation are involved in osteoclast differentiation.

Methods: MC3T3-E1 cells or MLO-Y4 cells were seeded on 3D scaffold and grown to 70–80% confluence. The cells were exposed to pressure of 1.5 MPa for 1 h at 37°C consisting a hydrostatic pressure system. After cultivation, the cultured media harvested and then isolated then centrifuged at 8,000 ×*g* for 30 min at 4°C to remove cell debris. The extracellular exosomes were pelleted in a final ultracentrifugation at 100,000 ×*g* for 1 h at 4°C. Pelleted exosomes were resuspended in PBS and ultracentrifuged again. The size distribution of exosomes was examined using a NanoSight Tracking Analysis LM20 System. The amount of osteoclast differentiation was estimated by TRACP staining. The MLO-Y4 cell vesicle membrane and vesicle internal protein profiles were analysed by nano-LC-MS/MS based shotgun proteomics.

Results: The vesicles isolated from mechanical stress-loaded MC3T3-E1 cells facilitated the mechanical stress-loaded osteoblast differentiation, but no effect against normal MC3T3-E1 cells. Though the vesicles isolated from mechanical stress-loaded MLO-Y4 cells had no effect against osteoblast differentiation, these vesicles significantly induced osteoclast differentiation.

To characterize the mechanisms by which mechanical stress-loaded MLO-Y4 cell vesicles induces osteoclast differentiation in murine macrophage RAW264 cells, we analysed vesicle membrane and vesicle internal proteins by nano-LC-MS/MS-based shotgun proteomics. As a result, Protein X was only detected in mechanical stress-loaded MLO-Y4 cell vesicles.

Summary/Conclusion: Our data indicated that mechanical stress-loaded MLO-Y4 cells vesicles are acting as one of osteoclast differentiation mechanisms. Now, we are further investigating whether Protein X is involved in osteoclast differentiation.

Funding: This work was supported by a Grant-in-Aid for Scientific Research (C) [No. 18K11019] from Japan Society for the Promotion of Science (JSPS).

PT01.06

A label-free aptasensor for electrochemical detection of gastric cancer exosomes

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Introduction: Emerging evidence indicates exosomes derived from gastric cancer cells enhances tumour migration and invasion through the modulation of tumour microenvironment. Here we represent a label-free electrochemical aptasensor for specific detection of gastric cancer exosomes. This platform contains an anti-CD63 antibody modified gold electrode and a gastric cancer exosome specific aptamer. The aptamer is linked to a primer sequence which is complementary to a G-quadruplex circular template. The presence of target exosomes could trigger rolling circle amplification and produce multiple G-quadruplex units. This HRP mimicking DNAzyme could catalyses the reduction of H_2O_2 and generate electrochemical signal. This aptasensor exhibits high selectivity and sensitivity towards gastric cancer exosomes with a linear response range from 4.8×10^3 to 4.8×10^6 exosomes/mL. Therefore, we expect this electrochemical apatasensor to become a useful tool for the early diagnosis of gastric cancer.

Methods: First of all, several gastric cancer cell or cancer overexpressed protein aptamers were screened in order to select gastric cancer exosome specific aptamer. Then different kinds of exosomes were captured in the anti CD-63 antibody modified gold electrode. Among these exosomes, only gastric cancer exosomes could trigger RCA to achieve the generation of large amount of G-quadruplex units. The products were then incubated with hemin to form hemin-G-quadruplex structures and catalysed H_2O_2 system to produce electrochemical signal. The aptasensor was also validated in terms of the linearity and repeatability to demonstrate its potential in practice.

Results: Anti-CD63, which can bind to the exosome surface marker was used as the capture probe. And the joint effects of hemin/G-quadruplex DNAzyme towards H_2O_2 reduction and signal amplification produced by RCA reaction was used to generate significantly strong electrochemical and colorimetric response.

Summary/Conclusion: In this work, we developed an electrochemical and colorimetric aptasensor for specific detection of gastric cancer exosomes. A specific gastric cancer exosome aptamer was selected and used as the detection probe. The aptasensor exhibits specificity towards target exosomes and high sensitivity.

PT02: EVs in reproduction and pregnancy
Chairs: Nanbert Zhong, Qi Chen
Location: Level 3, Hall A

15:30–16:30

PT02.01

Placenta extracellular vesicles: a potential protective role against oxidative damage

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Introduction: Extracellular vesicles (EVs) are lipid-enclosed packages of cellular contents including RNAs, protein and DNA that are produced by all eukaryotic cells to facilitate intercellular communication and regulation. Upon reaching their target cells, EVs may deliver their cargo and can induce signalling to alter the behaviour of target cells. During pregnancy, a large number of EVs are extruded from placenta (a foetal organ) into maternal circulation. Placental EVs are implicated in maternal immunosuppression and tissue repair. In this study we investigated whether placental EVs can prevent cell damage.
Methods: EVs were isolated from first trimester placental explants (range from 8–12 weeks of gestation) and separated into micro- and nano-EVs by differential centrifugation. Human endometrium epithelial cells (HEE) were cultured for 18 h in the presence or absence of placental micro- or nano-EVs. After removal of excess EVs by washing with PBS, HEE cells were treated with 1 mM H_2O_2 for 30 min and then the H_2O_2 was removed by washing. The culture was continued for 18 h and proliferation of HEE was measured by Alamar Blue assay. The expression of H2AX, a marker of DNA damage in HEE cells was measured by IHC.
Results: The proliferation of HEE was significantly reduced when HEE cells were treated with 1 mM H_2O_2. However this reduction of proliferation was significantly reversed by pre-treatment with either micro- or nano-placental EVs. In addition, the expression of H2AX was higher in HEE cells that had been treated with 1 mM H_2O_2, but higher expression of H2AX was reduced in HEE cells that had been pre-treated with either micro- or nano-EVs.
Summary/Conclusion: In this study, we found that pre-treatment with placental EVs can reduce the adverse effects of H_2O_2 on HEE cell proliferation death and DNA damage. Our data suggest placental EVs have the ability to protective cells against oxidative damage. In pregnancy this property of placental EVs may assist the function of maternal cells that are exposed to increased oxidative stress.

PT02.02

Maternal serum miRNA biomarkers for detection of placenta accreta

Rebecca R. Adami[a], Louise Laurent[b], Victoria Fratto[c], Srimeenakshi Srinivasan[a], Cuong Tran[a], Peter DeHoff[a], Melissa Westermann[d], Allison O'Leary[e], Deborah Wing[d] and Gladys Ramos[a]

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Introduction: Failure to diagnose placenta accreta spectrum (PAS) prior to delivery is associated with worse outcomes. However, use of ultrasound and magnetic resonance imaging for this diagnosis is costly and imprecise. We hypothesize that levels of specific cell-free miRNAs in the maternal blood will differ among women with PAS, placenta previa and normal placentation.
Methods: Women with suspected PAS, previa or normal placentation were prospectively recruited at three academic centres in the UC foetal Consortium. PAS was confirmed by pathologic evaluation. Maternal serum was collected antenatally, and total RNA was extracted, subjected to small RNA sequencing, and mapped to the miRBase human miRNA database. Groupwise differential expression analysis identified 13 candidate miRNAs, which were used to generate a support vector regression model for classification. The small RNA sequencing results for these candidate miRNAs were validated using qPCR.
Results: 60 women were recruited: 18 PAS, 15 placenta previa and 27 normal placentation. The median gestational age at sample collection was 30w3d (IQR 28w–33w) and did not differ among groups (p = 0.13). The abundance of total miRNA reads as a percentage of all reads in the small RNA sequencing data was highest among women with PAS and lowest in normal placentation. Thirteen differentially expressed candidate

miRNAs were identified. Support vector regression accurately classified samples into the three categories.
Summary/Conclusion: The percent total miRNA was significantly higher in maternal serum in cases of PAS compared to normal placentation. Thirteen candidate miRNAs were differentially expressed among groups and were used in a training model to accurately classify samples. Our results suggest that maternal serum miRNAs have the potential to serve as biomarkers for accurate antenatal diagnosis of PAS. Studies in a larger independent cohort are needed for validation of these results.

PT02.03

Effects of medium term storage on placental extracellular vesicles

Larry Chamley, Julie Wang and Cherie Blenkiron

The University of Auckland, Auckland, New Zealand

Introduction: Studies on the function of isolated extracellular vesicles (EVs) are growing exponentially. However, as yet there is no consensus on how best to store EVs. We hereby conducted a term study to examine the stability of various cargos carried by placental EVs when stored at 4°C.
Methods: First-trimester placental tissues were cultured for 24 h in medium supplemented with fluorescent cell tracker CMTPX (1 μg/mL). Debris was removed by centrifugation at 2000 ×*g*. Micro EVs were harvested by centrifugation at 20,000×*g* and subsequently nano-EVs were harvested following centrifugation at 200,000×*g*. The EVs were resuspended in PBS then aliquoted and stored at 4oC. CMTPX signal strength was examined by flow cytometry (AriaII) weekly. DNA was extracted, fortnightly, using Purelink Genomic DNA kit and measured using a Qubit dsDNA assay; and total proteins were isolated, fortnightly, with RIPA and quantified using BCA assay.
Results: The proportions of micro and nano-EVs showing similar intensity of CMTPX signals did not change significantly for 3 months ($n > 5$) but an inconsistent and sample-dependent decline was observed thereafter. In contrast, the DNA content of EVs was stable for only 2 weeks. DNA quantities extracted from micro and nano-EVs declined by 40% and 60%, respectively, at week four compared to DNA extracted from freshly isolated EVs and thereafter remained stable until 8 weeks. Total protein in micro EVs was stable for 2 months. Whereas there was a 20% decline in the total protein extracted from nano-EVs by week 2 but levels remained stable thereafter. Finally, the corresponding placental tissues also stored at 4oC and processed in parallel showed continuous decline in both DNA and protein quantities.
Summary/Conclusion: The CMPTX label incorporated into placental EVs may be stable for 3 months when stored at 4oC. However, the DNA of both micro and nano-EVs was less stable with a rapid decline upon storage. There was a marked difference in the stability of EV-associated protein with the protein content of nano-EVs being less stable than that of micro-EVs. Notably the total protein content of placental micro-EVs was remarkably stable when the EVs were stored at 4oC. Further work is required to assess the intactness/functionality of placental EVs after storage.
Funding: Marsden Fund of the Royal Society of New Zealand

PT02.04

Deciphering embryo-maternal communication; the dynamics of first contact between progenitor and progeny

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Introduction: Failure of implantation has long been identified as a major challenge of assisted reproductive technologies. It is hypothesized that the embryo alters the endometrium to elevated receptivity by embryo-maternal cross talk. In previous communications, we have shown that RNA originating from JAr (analogue for trophoblast) cell line, packaged in extracellular vesicles (EVs) are transferred to RL95–2 (an analogue for endometrium) cell line and induce alterations in specific endometrial Zinc Finger Protein 81 (ZNF81) transcript. The objective of the current study was to test the hypothesis that only EVs from viable embryo alter ZNF81 transcript in the RL95-2 cell line.
Methods: Human embryos were produced by classic in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). They were cultured individually for 20 h in Fert™ media (day 1), 48 h (day-3) in Cleav™ media and additionally 48 h in Blast™ media (day-5). At day-3, embryos with equal size blastomeres and no fragmentation were considered as normal. At day 5, embryos with identifiable inner cell mass, trophoblast and blastocyst cavity were considered normal while embryos forming mass of degrading cells were considered degraded. Conditioned media was collected from 6 normal day-3 embryos (three of which degraded by

day 5), day-5 normal (n = 3) and degraded (n = 3) embryos, Cleav™ and Blast™ media. EVs were isolated using a sequential centrifugation and size-exclusion chromatography. A monolayer of RL95-2 cells (analogue for endometrium) was treated with isolated EVs. The change of gene expression of ZNF 81 and control genes (beta-actin, beta-2-microglobulin) in RL95-2 cells were measured using qPCR with absolute quantification.

Results: Results exhibited that EVs derived both from day-5 normal blastocysts and day-3 embryos that undergo normal development significantly downregulated ZNF 81 expression in endometrial cells compared to untreated controls, cells treated with Cleave™ and Blast™ media EVs, cells treated with day-5 degraded embryos and day-3 embryos degrading on day-5 EVs. Control genes did not exhibit a significant change of expression.

Summary/Conclusion: RL95-2 cells respond in different manners to EVs from normal and degraded human embryos. These findings can facilitate development of biomarkers for differentiating viable and degraded embryos at early stages after IVF.

PT03: EV Nucleic Acid Biomarkers
Chairs: Louise Laurent; Guoku Hu
Location: Level 3, Hall A

15:30–16:30

PT03.01

Circulating exosomal miRNAs as potential biomarkers for evaluation of preterm brain injury

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Introduction: Insults such as oxygen deprivation occurring *in utero* or during delivery have profound consequences on the neurological outcome of premature infants. This is a serious clinical problem, because treatment is a time-critical emergency and should be commenced within 6 h following injury. However, we simply do not know which preterm infants to treat due to the lack of sensitive biomarkers. Using our foetal sheep model of preterm brain injury, we sought to isolate exosomes from foetal plasma to establish whether they contain miRNA biomarkers that are associated with clinically significant neurologic outcomes.
Methods: Chronically instrumented singleton foetal sheep at 0.7 gestation (term 145 days) received asphyxia induced by umbilical cord occlusion for 25 min. Size-exclusion chromatography (qEV) was performed for isolation and purification of extracellular vesicles (EVs) from plasma collected 4 h after occlusion from asphyxia (n = 10) and sham control (n = 10) foetuses. EV fractions were assessed for purity and quantity by nanoparticle tracking analysis and western blot against major EV protein markers. For biomarker identification, miRNA expression profiles from plasma EV fractions were determined by Affymetrix v4 microarrays.
Results: Umbilical cord occlusion was associated with significant brain injury to areas commonly affected by asphyxia in preterm infants. Plasma EVs were characterised as rich in CD63 and HSP70, size ~100 nm, and with an exosome-like morphology by TEM. Profiling of EV-miRNAs revealed significant differences (log2 fold change > 2 or < −2 and p value < 0.05) between the asphyxia and sham control foetal groups. Strikingly, the majority of miRNAs differentially abundant with asphyxial-induced brain injury were less abundant, including miR-30b-5p, miR-30a-5p, miR-27a, let-7f, miR-223/3p, miR-221, miR-22-3p, miR-151–3p, miR-411–5p and miR-532 whereas only one miRNA (miR-455-3p) was more abundant.
Summary/Conclusion: To the best of our knowledge, this study is the first to determine the usefulness of plasma exosomal miRNAs as biomarkers for the prediction of preterm brain injury. Our data reveal a unique plasma-derived exosomal miRNA profile, which may aid the early diagnosis of preterm brain injury.
Funding: Neurological Foundation of New Zealand.

PT03.04

Identification and Verification of Differentially Expressed MicroRNAs in the plasma microvesicles for the Diagnosis of moyamoya Disease

Mi Jeong Oh[a], Eun Hee Kim[a], Yeon Hee Cho[b], Dong Hee Kim[c], Ji Hee Sung[b], Eun Kyoung Shin[a] and Oh Young Bang[d]

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Introduction: There is no well-recognized miRNA biomarker for accurately predicting outcome in the presence of moyamoya disease (MMD), a unique cerebrovascular occlusive disease of unknown etiology[1,2]. We performed a study of the significance of miRNAs expression in the plasma microvesicles (MVs) of MMD patients.
Methods: The plasma MVs were purified from 38 healthy donors, 22 intracranial atherosclerotic stenosis (ICAS) patients and 40 moyamoya disease (MMD) patients. Plasma MVs were isolated using ultracentrifugation. We perfomed miR expression analysis using miRNome miScript miRNA PCR Array. Specific miRNAs were validated using real-time polymerase chain reaction, with normalization to an exogenous control (cel-miR-39). The angiogenic effects were measured by over-expressing or inhibiting specific miRNAs.
Results: MiRNA profiles using miRNome miScript miRNA PCR array of three pooled plasma MV samples from patients with MMD, ICAS and controls revealed 222 differentially expressed serum miRNAs, including 115 upregulated and 107 downregulated miRNAs. In

an independent MMD cohort, qRT-PCR confirmed that miR-A was significantly upregulated. Hsa-miR-A in the MMD group exhibited greater performance than ICAS group (AUC 0.735) in ROC curve analysis. To select target genes of specific miRNAs, we performed computational miR target prediction analysis (TargetScan) and found the seed sequence of CAV1 3'-UTR interacting with hsa-miR-A. The deregulation of miR-A by the transfection of HUVECs with pre-miR-A was significantly decreased tube formation of HUVECs. In addition, miR-A inhibited tube formation by suppressing the expression of CAV1at the posttranscriptional level, respectively, resulting in defective angiogenesis and MMD pathogenesis.

Summary/Conclusion: Hsa-miR-A is markedly elevated in plasma MV of patients with MMD. The potential role of these microRNAs in the pathogenesis of MMD can contribute to find new diagnostic and therapeutic target of MMD.

Funding: This study was supported by a grant from the Korean Healthcare Technology R&D Project, Ministry of Health & Welfare (HI17C1256) and Basic Science Research Program, the Ministry of Science, ICT and Future Planning (2018M3A9H1023675).

PT03.05

Micro RNA- 451-5p in urinary exosomes for non-invasive monitoring of reno-protective response

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Introduction: MicroRNA-451 in urinary exosomes (UE) had been demonstrated as a sensitive predictor of kidney injury in diabetic rats by us. Here, we determined whether miR-451 in UE could also assess severity of kidney injury and/or reno-protective response to therapy.

Methods: Streptozotocin-induced (STZ, 50 mg/kg of body weight, i.p) type-1 diabetic rats were fed with normal chow diet (DM) or high cholesterol diet (DM + HCD), to increase the severity of nephropathy. Half rats in DM + HCD group were treated with atorvastatin (AT, 20 mg/kg body weight, DM + HCD + AT) for 8 weeks and rest remained untreated. After 8 weeks, urine was collected for exosomes-enrichment and albumin to creatinine ratio (ACR). Primary cultures of human proximal tubular cells (hPC) and their secreted exosomes were also studied. Taqman-based real-time qPCR was done for miR-451-5p.

Results: At the end of the study, DM + HCD had significantly higher ACR while lower renal miR-451 levels, relative to DM and DM + HCD + AT rats. In addition, a strong negative correlation of renal miR-451 was found with ACR. In contrast to kidney tissue, UE analysis revealed a positive correlation of miR-451 levels with ACR in these rats. Moreover, miR-451 in UE was significantly higher DM + HCD rats relative to DM and DM + HCD + AT rats. *In vitro* studies confirmed the direct effect of hyperglycaemia / mechanical on miR-451 expression.

Summary/Conclusion: Severity of kidney injury in rats is associated with lower renal miR-451 and higher UE miR-451. Statin attenuated renal injury and miR-451 levels in UE, while improving renal levels. MiR 451 analysis in UE may assess therapeutic response of reno-protective drugs non-invasively

Funding: Funded by ICMR and DBT, Govt. of India.

PT03.06

Circulating miR-451a is a useful biomarker for haemolysis

Yukichi Takada, Tatsuki Shibuta and Tsukuru Umemura

International University of Health and Welfare, Okawa City, Japan

Introduction: Red blood cells (RBCs) are circulating enucleated cells, and its main component is haemoglobin carrying O_2/CO_2. RBCs contain erythroid lineage-specific microRNA (miRNA), miR-451a. Biogenesis of miR-451a depends on the activity of Ago2, and also circulating miR-451a is mainly Ago2-bound, non-exosome miRNA. For utilizing circulating miR-451a for diagnosis of anaemia which is caused by destruction of RBCs (haemolysis), it is important to evaluate distribution pattern of circulating miR-451a.

Methods: We analysed 120 remnant serum samples obtained from routine blood drawing for laboratory testing. The study was done under permission of The Ethical Committies of International University of Health and Welfare and Kohoukai Takagi Hospital. Serum miRNAs were analysed using TaqMan microRNA assay kits and RT-qPCR (ABI 7500fast). Exosomes were obtained using the ultracentrifugation method and the precipitation method.

Results: We analysed 120 remnant serum samples obtained from routine blood. The sensitive Hb detection method using 414 nm absorbance (NanoDrop2000, ThermoFischer) showed that 100% of blood sampling had haemolysis. The levels of serum miR-451a also increased in all samples. Ultracentrifugation method and precipitation method

showed more than 90% of serum miR-451a is in the non-exosome fraction.

Summary/Conclusion: miR-451 is a unique miRNA which is in the non-exosome fraction. The measurement of serum miR-451a is a sensitive and specific biomarker for haemolysis.

Funding: This work was supported in part by a grant from the Japan Society for the Promotion of Science (JSPS KAKENHI Grant Number: JP17K09020).

PT03.07

Circumventing qPCR inhibition to improve amplification of exosomal miRNAs in preterm foetal sheep heparinised plasma

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Introduction: Exosomal miRNAs have been identified in plasma, which has led to an interest in their potential as biomarkers of neural injury responses in survivors of prematurity. The chronically catheterized preterm foetal sheep model is a uniquely versatile model to advance our understanding of the pathophysiological mechanisms underlying preterm brain injury and develop new therapeutic strategies. However, to ensure patency of catheters implanted into foetal vessels heparinised saline is infused continually. Heparin can confound detection of plasma miRNAs. Here we present an optimal procedure to collect and detect exosomal miRNAs in foetal plasma that improves sensitivity and performance of qRT-PCR.

Methods: Size-exclusion chromatography (SEC; qEV) and commercial ExoRNeasy were performed for isolation and purification of extracellular vesicles (EVs) on foetal plasma collected in K2EDTA tubes in which varying amounts of heparin or enoxaparin (0–160 IU/mL) had been added post-thawing. RNA was extracted from qEV fractions (miRNeasy), ExoRNeasy EVs, or from whole plasma (miRNeasy) and treated with or without heparinase I to remove contaminating heparin. Levels of endogenous miR-16 and let-7a were measured using qRT-PCR.

Results: Important differences in EV-miRNA abundance were observed between isolation methods. Heparinase I treatment improved detectability of the miRNAs in a dose-dependent manner in heparin/non-heparin spiked whole plasma and when isolated using the ExoRNeasy kit. Strikingly, SEC removed endogenous heparin from non-heparin spiked plasma collected from catheters infused with low-dose heparin and this effect was not improved by heparinise I. Further, SEC partially removed contaminating effects of heparin in heparin spiked whole plasma.

Summary/Conclusion: Treatment of foetal sheep plasma with heparinase I enables utilisation of qRT-PCR for reliable miRNA quantification. SEC also enables removal of inhibitory heparin and therefore should be considered as a standard step for isolation and detection of EV-miRNAs in foetal plasma.

Funding: Neurological Foundation of New Zealand.

PT03.09

A particle-based multiplex RT-qPCR for measuring circadian rhythm-associated genes

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Introduction: Biological clocks which regulate the expression of genes related to the circadian metabolism of living organisms are directly linked to human health and disease. Many studies also have suggested to analyse mental health issues such as depression and bipolar disorder through molecular analysis of circadian rhythm-associated genes recently. These genes are generally analysed by reverse-transcription real-time PCR (RT-qPCR) but conventional methods require considerable time, cost and amount of the sample to figure out multiple genes at the same time.

Methods: We introduce a sensitive and multiplex RT-qPCR using hydrogel particles. One of the primer pair used in RT which captures and reverse-transcribes the target RNA is immobilized in the particle. The other primer is stored not to react during RT and released when amplification begins. This strategy can help the RT process to avoid non-specific products as well as the amplification to occur effectively.

Results: In order to see RT-qPCR efficiency, PER3 RNA which is synthesized via *in vitro* transcription (IVT) of complementary DNA, and total RNA extracted from LNCAP cell were tested with serial dilution. As a result, they showed high amplification efficiency of 95% and 98%, respectively. In addition, the expression level of genes was successfully measured even from a single cell. Expression pattern of 8 circadian rhythm-associated genes was acquired from total

RNA of circadian rhythm-synchronized HeLa cells. In addition, exosomal RNAs were monitored same cells every four hours for 48 h in order to obtain molecular circadian rhythm cycles. Each gene showed unique pattern of expression.

Summary/Conclusion: For non-invasive diagnosis, clinical sample of human epithelial cells, saliva exosome and hair cells were confirmed particular patterns. Further study will emphasize to take a snap-shot of molecular circadian rhythm through the combination of expression levels of associated genes measured at a certain time point for diagnosis of the mood disorder.

PT04: EV Nucleic Acid Cancer Biomarkers
Chairs: Christian Preußer; Harry Holthofer
Location: Level 3, Hall A

15:30–16:30

PT04.01

Unveiling of extracellular exosomal miRNA profiles of breast cancer

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Introduction: In an era of precision medicine, biomarker discovery is indispensable for early detection, therapeutic efficacy monitoring and outcome prediction. MicroRNAs within patient serum exosome have emerged as significantly measurable biomarkers, which abundantly existed in the form of liquid biopsies, for several diseases, including cancers. They are essential regulators of global mRNA expression in cells. Aberrant regulation of miRNA can enables resulting in tumour initiation, drug resistance and metastasis in cancer. miRNA assays are convenient for large-scale studies covering multiple miRNA targets and realistic in screening across diverse breast cancer types for early detection or factors that drive cancer progression.
Methods: In this study, we collected patient serum samples from four major molecular subtypes: luminal A, luminal B, HER2+ and triple negative types, and breast cancer patients with benign tumour and ductal carcinoma in situ (DCIS). Microarray analysis of miRNA expression was utilized and unique serum miRNA signatures between non-cancer (including benign, DICS) and breast cancer patients were identified with differential expression analysis and the common features selection method – elastic net. All combinations of selected miRNAs were modelled with three different approaches, including generalized linear model (GLM), linear discriminant analysis (LDA) and support vector machine (SVM).
Results: To analyse more generally, we applied various normalization methods and got different outcomes after feature selection. All the selections were used to fit prediction models. After applying the filter criteria based on accuracy evaluation by 10-fold cross-validation, the top selected miRNAs showed the consistency of different prediction methods, and the union of these selections was used in the further modelling. The results confirmed that few miRNAs are enough to implement early detection at high accuracy.
Summary/Conclusion: Through identifying these heterogeneous compositions of the cancer cells, understanding of the molecular mechanisms underlying these identified biomarkers, which is essential in developing effective treatments and translational research, could be established.

PT04.03

Hypoxia may promote tumour aggressiveness and extracellular vesicle-mediated cell-to-cell communication in multiple myeloma

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Introduction: Hypoxia is one of important features of tumour microenvironment, and tumour cells under hypoxia can acquire aggressive characteristics including drug resistance. Thus, tumour progression and resistance to therapy are associated with hypoxic tumour microenvironment. Multiple myeloma (MM) is a neoplasm of bone marrow plasma cells. Bone marrow is hypoxic compared to other organs, thus, tumour aggressiveness of MM could be closely associated with hypoxic microenvironment. Extracellular vesicle is a small vesicles containing a wide range of functional proteins, mRNAs and miRNAs that are actively secreted via exocytosis. In this study, we investigated the effect of hypoxia on the EV formation of MM cells to identify the underlying mechanism of tumour aggressiveness in MM cells.
Methods: We conducted a long-term culture of MM cell lines under hypoxic conditions (1% and 2% of O2 for 4 weeks), and compared with same MM cell lines cultured under normal oxygen concentration (20%). The RNA expression profiles of MM cells under hypoxia were also compared with that of cultured cells under normal oxygen condition. The EV derived from MM cells was isolated using ExoQuick-TC solution and assessed by transmission electron microscopy, Nanoparticle tracking analysis and Western blot.

Results: The overexpression of HIF-1α was demonstrated in MM cells under long-term hypoxia, and the expression of stem cell markers were more increased in MM cells under hypoxic condition compared to normal oxygen concentration The RNA sequencing showed up-regulation of gene associated with production of EV in hypoxic cultured cells. When we measured EV from hypoxic cultured MM cells, the amount of EV was significantly higher in hypoxic MM cells than normoxic control group. To identify specific alterations associated with hypoxic MM cells, we profiled miRNAs derived from EV of hypoxic MM cell lines and those of normoxic MM cell lines. These results identified eight miRNAs with significantly different expression between MM cells – derived EV.

Summary/Conclusion: We demonstrated the characteristics of long-term hypoxic MM cell-derived EV. The EV-mediated cell-to-cell communication under hypoxia might be associated with the content of miRNA in MM cell-derived EV, and it might influence tumour aggressiveness of MM cells.

PT04.04

Deep sequencing identified serum exosomal miR-181a-5p as an indicator for bone-metastatic prostate cancer

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Introduction: Prostate cancer (PCa) is the most common male malignancy worldwide with high heterogeneity from tumorigenesis to metastasis. Although bone metastasis is the most critical metastatic event, at present, there has been no specific and accurate biomarker for its diagnosis or differentiation at an early stage of PCa. Given the fact that the profiling change of exosomal miRNAs can work as a biomaker for metastasis in multiple tumours, we seek to identify exosomal miRNAs in patient's serum as indicators for bone-metastatic PCa.

Methods: The profiling change of serum exosomal miRNAs in patients with either benign prostatic hyperplasia (BPH) or localized or bone-metastatic PCa was detected by miRNA-seq and miRNA-chip array, respectively. Prospective miRNAs were further confirmed using TaqMan miRNA assay in two independent validation cohorts of total 127 patients with either BPH or localised or bone-metastasic PCa. Logistic regression analysis was performed to evaluate the diagnostic association of candidates with bone metastasis. Accuracy estimate of each candidate for the diagnosis of bone-metastatic PCa was quantified using the area under the receiver-operating characteristic curve (AUC).

Results: By miRNA-seq and miRNA-chip array, we found four prospective exosomal miRNAs including miR-181a-5p with significant differences between localized and bone-metastatic PCa groups (p□0.05, fold change ≥1.5 or ≤0.5). In the validation cohorts, logistic regression analyses indicated that miR-181a-5p and miR-320a were significantly associated with bone-metastatic PCa. The AUC analyses identified miR-181a-5p as the best biomarker with the AUCs 93.1% for diagnosis of PCa and 73.9% for that of tumour bone metastasis.

Summary/Conclusion: Serum exosomal miR-181a-5p is a promising diagnostic biomarker for bone-metastatic PCa. Further validation is needed.

Funding: National Natural Science Foundation of China (81630073 to W-QG, 81874097 to Y-XF, 81672850 to BD, 81572536 and 81772742 to WX)

PT04.05

Exosomal miRNAs and proteins signature as prognostic biomarkers for early stage epithelial ovarian cancer

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Introduction: Epithelial Ovarian Cancer (EOC) is the leading gynaecological malignancy worldwide due to the limitations of current detection tests. The 5-year survival rate with early detection is 90% compared to 20% with late detection. Unfortunately, only 30% of the cases are detected early. Thus, it is essential to develop a novel and minimally invasive method to identify patients at an early stage. Exosomes have shown promise as biomarkers as they encapsulate vital information. Therefore, the aims of this study were to (i) determine the content of circulating exosomes at early stages of EOC, and (ii) to determine the prognostic performance of an early-ovarian cancer screening test to identify women at risk of developing EOC.

Methods: Exosomes were isolated from the plasma of patients with either benign disease (n = 50) or Stage I/ II EOC (n = 28), through differential centrifugation

and size exclusion chromatography. Exosomes were characterized using Nanoparticle Tracking Analysis, Western Blot and Electron Microscopy. Exosomal proteins were profiled using Liquid Chromatography–Mass Spectrometry (LC-MS/MS) and SWATH analysis. An Illumina TrueSeq Small RNA Library Prep kit was used for exosomal miRNA profiling. A binomial classification algorithm was generated using a boosted logistic regression analysis (WEKA machine learning software (ver 3.6.12)) of the results obtained from the benign and Stage I/II samples. The algorithm was built using 5 miRNAs and 5 proteins identified through circulating exosome profiling. The expression of specific miRNAs was confirmed using RT-qPCR to validate the miRNA sequencing results.

Results: miRNAs and proteins were identified as being differentially expressed across EOC progression. The algorithm that we built delivered discrimination between women with EOC (Stage I/II) compared to benign. The classification efficiency was assessed by ROC curve analysis (area under the curve (AUC) was 0.785 ± 0.091 (p = 0.0106)) with positive and negative predictive values of 75% and 76%, respectively.

Summary/Conclusion: We propose that the combined measurement of exosomal miRNAs and proteins might allow for the early identification of women with EOC, distinguishing between patients with benign disease and patients with Stage I/II EOC. Future directions involve the validation of the proposed miRNAs and proteins in a larger cohort.

Funding: OCRF.

PT04.06

Circulating Extracellular vesicle (EV)-encapsulated microRNAs as a biomarker of breast cancer

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Introduction: Early detection of breast cancer is the key to improve patient survival. There is currently no robust biomarker available to detect breast cancer. Extracellular vesicle encapsulated microRNAs (EV-miR) provide novel potential in this field. EVs are tiny nanoparticles released by all cells in the body that contain various bioactives including miRNA, believed to reflect the characteristics of the parent cell. Evidence suggests that tumour associated EVs have a distinct miRNA expression profile from normal cells, and could hold novel biomarker potential.

Methods: This study aimed to determine the circulating EV-miR profile of tumour-bearing animals compared to healthy controls, and relate this to the EV-miR profile secreted by tumour cells *in vitro*. EVs were isolated from the supernatant of HCC-1954 breast cancer cells expressing luciferase (HCC-luc). EVs were also harvested from healthy BALB/c nude mice or those bearing mammary fat pad HCC-luc tumours. Serum and media EVs were isolated by differential centrifugation, followed by microfiltration and ultracentrifugation. EVs were characterised by Nanoparticle Tracking Analysis (NTA), western blot and Transmission Electron Microscopy (TEM). Next-Generation Sequencing (NGS) targeting miRNA was preformed to compare the HCC-luc EV-miR profile *in vitro* and *in vivo*.

Results: EVs were successfully isolated and demonstrated to express CD63, CD9 and CD81. Using NTA, size distribution was confirmed to be of the predicted range of 30–120 nm. A range of miRNA was detected in the HCC-luc EVs *in vitro*. Interestingly, nine of these miRNAs were present at significantly higher levels in the EVs than the cells from which they were released, e.g. miR-184 and let-7c. Initial analysis revealed that a number of miRNAs packaged into EVs by HCC-luc cells *In Vitro* were also detectable in circulating EVs isolated from tumour-bearing animals.

Summary/Conclusion: EVs are thought to represent a fingerprint of the cell from which they are released, and hold great potential as biomarkers for breast cancer detection. Further understanding of miRNA trafficking and transfer in EVs will shed light on their true potential in the cancer biomarker and therapeutic setting.

Funding: I am funded by the National Breast Cancer Research Institute (NBCRI).

PT04.07

Role of exosomal microRNA as a biomarker for extranodal NK/T-cell lymphoma

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Introduction: Extranodal natural killer (NK)/T-cell lymphoma (ENKTL) is one of aggressive subtype of non-Hodgkin lymphoma, and all tumour cells are inarguably infected with Epstein–Barr virus. The cell to cell interaction and association with tumour microenvironment could be important for this disease entity. Exosomes are small membrane vesicles of 30–150-nm diameter that plays an important role in the tumour microenvironment, and they are actively secreted by

most cell types, including cancer cells. In particular, the intra-exosomal microRNA is known as being important for intercellular communications. However, the clinical significance of exosomal miRNAs in ENKTL has not been examined. Thus, we characterized exosomal miRNAs in ENKTL and analysed their effect on the outcomes of patients.

Methods: We isolated exosomes from ENKTL patient serum and lymphoma cell lines using ExoQuick and analysed by transmission electron microscopy, Nanoparticle tracking analysis (NTA) and Western blot. We performed exosomal microRNA profiling via the nCounter miRNA expression assay on exosomes from 45 ENKTL patients and lymphoma cell lines.

Results: We isolated and characterized exosomes from NKTL patient serum and cell lines using ExoQuick, and analysed by TEM, NTA and Western blot. The serum-derived exosomes had a diameter of 95.84 ± 11.37 nm and exosome concentrations ranged from 0.25 to 14 × 1012/mL. We verified exosomes morphology and size using TEM, and detected exosomal markers, including Alix, and CD63 by western bolt. We performed miRNA microarrays to compare exosomal miRNAs of patients with ENKTL having good and bad prognosis. As shown in the microarray results, we found various miRNAs that were differentially contained in the serum – derived exosomes of NKTL poor relative to good subjects. These results identified 30 miRNAs with significantly different expression between NKTL samples. Five of these miRNAs were up-regulated and 25 ware down-regulated in the serum-derived exosomes of NKTL bad compared to the good subjects (p value < 0. 05). We identified two exosomal miRNA signatures, has-miR-320e and miR-4516, that were associated with poor outcomes with regard to OS and PFS.

Summary/Conclusion: Our study provides that exosomal miRNA, miR-320e and miR-4516, may serve as potential diagnostic and prognostic biomarker in NKTL.

PT04.08

Cancer-derived exosomes enriched from patient plasma strongly mirror parent tumour and enable subtyping of early stage breast cancer via liquid biopsy

Christine Coticchia[a], Robert Kitchen[b], Sudipto Chakrabortty[b], Douglas Roberts[a], Lisa Bedford[c], Sunita Badola[c], Sylvie Vincent[c], Seth Yu[B] and Johan Skog[d]

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Introduction: Tumour-derived molecular signatures of breast cancer (BCa) have accelerated personalized medicine as prognostic and predictive indicators leading to improved clinical outcomes. Currently, molecular profiling is performed on biopsied breast tumour tissue but our goal of “liquid biopsy” is to obtain disease-relevant genetic material non-invasively by capturing exosomes, cfDNA, or protein from bodily fluids. Unfortunately, a major limitation of liquid biopsy stems from the scarcity of disease-relevant material compared to background. Here we describe an enrichment process in plasma capable of isolating cancer specific exosomal subpopulations originating from early stage breast tumours.

Methods: Tumour-specific surface markers on exosomes were targeted and enriched from plasma obtained from stage I/II ER positive / HER2 negative BCa patients and age-matched controls. RNA-sequencing was performed on total RNA isolated from 15 BCa tumour tissues (FFPE) and 15 patient-matched plasma exosome samples (with and without exosome enrichment). We also sequenced RNA from 12 healthy breast tissues (FFPE) and plasma exosomes from 10 healthy post-menopausal women (with and without tumour exosome enrichment). RNA-seq data were used for gene-level differential abundance analysis.

Results: Tumour-derived exosome enrichment was observed in 63% of the BCa patients with detectible levels of the target antigens in their plasma. RNA-seq gene expression profiles of these enriched exosomes were highly correlated with those of the breast tumour FFPE samples. Tumour-enriched exosomal RNA abundance clustered most tightly with the FFPE tissue derived from the same patient; even more so than BCa FFPE samples correlated to each other. The strength of the correlation between BCa enriched plasma exosomes and matched patient tissue was sufficient to enable correct tumour subtyping (by both PAM50 & IntClust gene targets) using only the enriched plasma exosomal RNA.

Summary/Conclusion: Tumour-specific exosome enrichment improved plasma-derived exosomal RNA signal to noise and revealed RNA profiles that closely reflect the donor tumour, thus enabling the detection and characterization of early stage breast cancers.

PT04.09

Exosomes: the same team for hepatocellular carcinoma development on the background of HCV and ergotism?

Alisa Petkevich, Alexandr Abramov, Mohamed Kadle and Vadim Pospelov

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Introduction: Hepatocellular carcinoma (HCC) may be caused by a wide variety of reasons, two possible of them are hepatitis C virus infection (HCV) and alkaloids contained in the ergot (Claviceps). Anyway, not all of the individuals infected with HCV or living in regions endemic for ergot develop HCC so it is reasonable to develop biomarker panel for identification of risk groups for HCC. Exosomes seem to be an ideal source of such biomarkers as far as they contain exactly the information molecules packed by cells during its physiological (or pathological) functioning.

Methods: 48 plasmas of patients with HCC from Somalia (from a region with a high degree of ergot alkaloides in food), and 18 plasmas of HCC (Russia) on the background of cirrhosis due to HCV. Exosomes were isolated from plasma by differential ultracentrifugation following free-flow electrophoresis. MiRNA let-7a-5p, −224-5p, −106b-3p, −126-5p, −122-5p, −16-5p and −34a-5p were determined in exosomes by qPCR-RT. Same free miRNA from plasma were determined. PD-L1 expression was assessed on the surface of exosomes by TEM and HR-FCM. PD-L1 expression was also assessed on the surface of exosomes isolated from plasma of healthy donors (n = 8).

Results: There was a slight difference in exosomal miRNA profile of plasma from HCC on the background of HCV and on the background of HCV and living in ergot region. PD-L1 expression on the surface of exosomes from HCC plasmas were higher (MV 35,8% for both HCC groups, MV 5% for healthy donors group). Plasma free miRNA profiles were different inside every HCC group.

Summary/Conclusion: According to our results, exosomal miRNA identification in HCC patients seem to be more accurate than plasma free miRNAs, further research is needed in order to identify whether it is reasonable to use both free and exosomal miRNAs. The difference in miRNA profiles of HCC patients on the background of HCV or alkaloids of ergot may allow supposing different epigenetics dysregulation happen in HCC depending on the trigger factor.

PT04.10

Exosomal sorting of circRNA promotes cancer progression and serves as a novel biomarker for gastric cancer

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Introduction: Exosomes are critical mediators of intercellular communication and promising biomarker for cancer. However, whether the release of exosomes has an effect on donor cells has not been well investigated. In this study, we aimed to identify the clinical values of exosomal circRNAs in gastric cancer (GC). Meanwhile, we explored the biological roles and mechanisms during GC cells selectively sorting exosomal circRNAs.

Methods: Database circ2Traits and starBase v2.0 were used to screen potential GC related circRNAs and validated their expression levels in over 50 paired serum or exosomes from GC patients and healthy volunteers, or 100 cancer tissues and adjacent normal tissues from GC patients. Receiver operating characteristic curve, clinicopathological features analysis and overall survival (OS) and disease-free survival (DFS) curve were made to evaluate the clinical relevance of these circRNAs and select circ1477 as potential biomarker for further studies. Circ1477 overexpression and knockdown experiments were conducted to assess the effects on GC progression *in vitro* and *in vivo*. RIP, luciferase assay, RNA FISH and rescue experiments were applied to demonstrate its molecular mechanism.

Results: We found that the level of circ1477 in serum or serum exosomes of GC patients was significantly higher than that in healthy volunteers. Whereas, the level of circ1477 in cancer tissues and was remarkably lower compared to adjacent normal tissues of GC patients, which was associated with lymph node metastasis and prognosis. Also, the expression of circ1477 was observed to be decreased in GC cell lines while increased in their derived exosomes, suggesting that circ1477 could be selectively sorted into exosomes by GC cells. Furthermore, cytoplasmic circ1477 could suppress the migration and invasion of GC cells acting as miRNA sponges while knockdown of it could reverse these effects.

Summary/Conclusion: Taken together, our findings indicate that tumour suppressive circ1477 could be selectively sorted into exosomes to promote tumour progression and serve as a potential biomarker for gastric cancer.

Funding: National Natural Science Foundation of China: (81572075); Technology Development Project of Jiangsu University (20180361)

PT04.11

Development of non-invasive tests for prostate cancer

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Introduction: Prostate cancer (PC) is the most common non-skin cancer in males and is fast becoming the most frequently diagnosed cancer in men. Despite significant advances in diagnosis and treatment PC remains a leading cause of cancer deaths. Analysis of PSA in blood has long been used for early diagnosis and monitoring but is flawed by low sensitivity and a high rate of false positives, with negative health consequences including the overtreatment of many indolent prostate cancer tumours. Caldera Health is developing non-invasive liquid biopsy tests for prostate cancer to improve upon and replace the controversial serum PSA test.

Methods: Through a series of clinical studies, Caldera Health has identified promising RNA biomarkers for PC diagnosis. Preliminary experiments indicated that in urine a far greater proportion of prostate RNA is localised in extracellular vesicles (EVs) than in cellular material. A simple and reliable process was optimised to concentrate urinary EVs and a novel method was developed to specifically isolate the EV's of prostatic origin with high efficiency. Subsequently a clinical study was performed using qRT-PCR to quantify RNA biomarkers in approximately 300 urine samples collected from men scheduled for prostate biopsy tests. The clinical study participants provided informed consent and the study was approved by recognised medical ethics committees in New Zealand and Australia.

Results: Comparison of the qPCR data for prostate, bladder and kidney-specific genes indicated our prostate vesicle isolation method successfully reduces contamination with vesicles from both kidney and bladder. The clinical study data was used to develop accurate prostate cancer diagnostic models.

Summary/Conclusion: Caldera Health has identified EV RNA biomarkers associated with prostate cancer and developed a novel method to specifically isolate prostate-derived EVs from urine. We have tested multiple biomarkers and developed gene signatures identifying prostate cancer with high sensitivity and specificity.

PT05: EV Biogenesis
Chairs: Imre Mager, Hollis Cline
Location: Level 3, Hall A

15:30–16:30

PT05.01

Uncovering the role of heparan sulphate proteoglycans in extracellular vesicle biogenesis: potential tools for improved therapies

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Introduction: Many cell types deliver therapeutic effects by secreting extracellular vesicles (EVs). Therefore, EVs could be used as an alternative approach to cell-based therapies, overcoming many cell-associated challenges. EVs could be optimised to generate potent therapies through manipulating the mechanisms driving EV biogenesis. We aim to prove this concept by altering the heparan sulphate (HS) chains found on syndecan, a key component in the syndecan-syntenin-ALIX mechanism. We predict that HS is involved in cargo selection due to its ability to form interactions with a wide range of factors. In addition, the structure of HS influences the activity of heparanase, a regulator in the rate of EV production. Therefore, structural alterations to HS could allow the cargo (thus therapeutic activity) to be modulated whilst simultaneously increasing EV yields.
Methods: MCF-7s mutated to alter expression of HS biosynthetic enzymes were generated using CRISPR-Cas9. Wild type and mutant MCF-7s were cultured in bioreactors using media containing EV-depleted Knockout Serum Replacement. EVs were isolated by differential ultracentrifugation and characterised using Transmission Electron Microscopy (TEM), Nanoparticle Tracking Analysis (NTA) and Western Blot.
Results: A FACS-based method has been developed to characterise and sort EVs based on their displayed HS. The cargo and functional activity of the sorted populations was then assessed. Since heparanase influences EV production rates, MCF-7s were incubated with a heparanase inhibitor (OGT2115). Subsequent alterations to soluble, cellular and vesicular HS composition was analysed by fluorescent labelling and SAX-HPLC identification. EV size and concentration was assessed using TEM and NTA.
Summary/Conclusion: Optimising EVs may generate highly efficacious and cost-effective treatments in comparison to those based on the producer cell line. Alterations to the HS structures on syndecan could be an ideal method for optimisation.
Funding: This PhD project is funded by EPSRC and MRC.

PT05.02

Augmentation by GnRH of ectosome containing annexin A5 formation by blebbing of pituitary gonadotropes and its biological effect

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Introduction: We have demonstrated that gonadotropin releasing hormone (GnRH) stimulates the synthesis of annexin A5 (ANXA5), a member of annexin family protein, in the pituitary gonadotropes and ANXA5 augments GnRH stimulation of gonadotropin secretion. It is, however, obscure how ANXA5 augments gonadotropin release at gonadotropes. As ANXA5 was demonstrated both in and out of cells, in the present study, we examined translocation of ANXA5 in response to GnRH stimulation in relation to the release of luteinizing hormone (LH).
Methods: Rat pituitary tissues, primary pituitary cells and LβT2 gonadotrope cells were used. The conditioned medium was sequentially centrifuged at 20,000 ×*g* and 110,000 ×*g* to obtain ectosome and exosome respectively. Immunochemistry for ANXA5 and LHβ were performed. Transmission electron-microscope (TEM) was also used.
Results: GnRH agonist (GnRHa) administration showed the formation of blebs containing ANXA5 on LβT2 cells and primary pituitary cells after only 10 and 30 min incubation. Hemi-pituitary gland was cultured with GnRHa and TEM showed that the boundary of GnRHa stimulated gonadotrope-like cell became obscure with many bubble like particles after 30 min incubation. The 20,000 ×*g* and 110,000 ×*g* particles

were increased by the GnRHa treatment. ANXA5 was detected dominantly in 20,000 ×*g* pellet after treatment with GnRHa. It increased until 180 min. ANXA5 in 110,000 ×g pellet was also shown at 180 min. GnRHa treated 20,000 ×g particulate fraction significantly stimulated LH release in a dose dependent manner. Extracellular vesicle fraction prepared from plasma of one-week ovariectomized rats, in which GnRH secretion was expected to be augmented, showed significant increase of ANXA5 in the 20,000 ×*g* pellet. The blebbing induced by GnRH was inhibited by H89, protein kinase A inhibitor. It is suggested that Gαs signalling is necessary for GnRH stimulation of blebbing.
Summary/Conclusion: Present study clearly demonstrates a hormonal regulation of ectosome formation and a novel mechanism of cell–cell communication by means of ANXA5 including ectosome.

PT05.03

Investigation into a novel role for the prolyl isomerase cyclophilin A during Extracellular vesicle signaling in cancer

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Introduction: Extracellular vesicles (EVs) released from cells mediate local and systemic cell–cell communication via the horizontal transfer of functional protein, DNA and RNA into recipient cells. Evidence reveals that tumour-derived EVs mediated intercellular communication between tumour cells and normal cells within the tumour microenvironment to initiate metastatic niche formation. Thus, disruption of EV-mediated tumour-niche interactions is a novel strategy for metastasis prevention. However, significant challenges in EV biology must be overcome for the translation of EVs into the clinic; in particular, in understanding their biogenesis and mechanism of action within the tumour microenvironment. The prolyl isomerase Cyclophilin A is overexpressed in a large variety of cancers and is associated with an aggressive phenotype of metastasis and chemoresistance. Unpublished data from our lab revealed that loss of CypA expression significantly reduced tumour growth and metastasis *in vivo* supporting a role in tumour progression. In this study, potential functions of CypA in EV biology and function are investigated.
Methods: EV Isolation: Differential Ultracentrifugation, Optiprep Density Gradient

EV characterization: Nanosight Tracking Analysis, Flow cytometry, Transmission Electron Microscopy, Mass spectrometry EV identification: Western Blot, Co-immunoprecipitation
Results: CypA is found to be enriched in cancer-derived EVs in a range of solid and haematopoietic malignancies. In addition, CypA is predominantly found in EVs within a specific density range. Moreover, homozygous loss of CypA expression reduces the number of EVs within a specific size range. Investigation of CypA interacting proteins by mass spectrometry reveals potential functions in EV cargo loading.
Summary/Conclusion: This study reveals a potential role for CypA in EV biogenesis, and highlights its potential as a novel EV target for the prevention of tumour progression. Significance of this study is that CypA could be a potential target for EV release. This work contributes to the understanding of CypA-dependent EV subtype for its biology and function during cancer metastasis and may reveal novel strategies for the generation of targeted EV subtype therapeutics.
Funding: UCD-CSC Scholarship (not include travel funding).

PT05.04=OWP2.12

Identification of a protein that presumably controls bacterial vesiculation in response to the extracellular environments

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Introduction: Many bacteria utilize extracellular membrane vesicles (EMVs) for survival in their growing environments through communication with others, pathogenesis and biofilm formation. Therefore, the amounts and the components of EMVs should be tuned in response to the conditions. Although several vesiculation mechanisms are suggested, little is known how bacteria control vesiculation in response to the environments. A bacterium *Shewanella* sp. HM13 has 9-fold higher lipid-secretion capability in EMV fractions than *Escherichia coli*, and its EMVs contain a major protein (P49), which is not required for vesicle production. We used mutant EMVs that lack P49 to identify minor components of EMVs that may control vesiculation.
Methods: EMVs were subjected to 2D gel-based proteomics by peptide mass fingerprinting. Within the identified proteins, the function of a sensor protein homolog, HM1275, was analysed by swarming assay and lipid-staining to quantify EMVs produced in various media. Changes in the number of EMVs

depending on culture media were quantified by tunable resistive pulse sensing method.

Results: A protein with a PAS domain and a methyl-accepting chemotaxis protein (MCP) sensing domain, HM1275, was identified in the EMVs. Although some MCPs are related to flagellar motility by binding some attractants, the flagellar motility of Delta-hm1275 was not significantly different from that of WT. Although the amounts of EMVs produced by WT were increased in response to the concentration of casamino acids in poor nutrient medium, those by Delta-hm1275 were not.

Summary/conclusion: A putative sensor protein, HM1275, was identified in EMVs and may recognize the extracellular environments by binding signal molecules in casamino acids to control vesiculation. Although further studies are required to reveal the signals and the sensing pathways, the results obtained in this study indicate that bacterial vesiculation is controlled by extracellular environments, and artificial control of vesiculation with extracellular signals would be useful in applications such as suppression of vesicle-dependent pathogenicity.

Funding: Japan Society for Promotion of Science Research Fellowship for Young Scientists

PT05.05=OWP2.13

Prokaryotic BAR domain-like protein BdpA promotes outer membrane extensions

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Introduction: Bin/Amphiphysin/RVS (BAR) domains belong to a superfamily of membrane-associated coiled-coil proteins that influence membrane curvature. BAR domains are ubiquitous in eukaryotes and associated with membrane curvature formation, vesicle biogenesis/trafficking, protein scaffolding and intracellular signalling. While advances in protein domain prediction have facilitated the identification of several BAR domain proteins, they have yet to be characterized in bacteria. Here, we identified a putative BAR domain-containing protein enriched in the outer membrane vesicles (OMVs) of *Shewanella oneidensis* MR-1, a dissimilatory metal-reducing bacteria known to produce outer membrane extensions (OMEs) that are suspected to facilitate long distance extracellular electron transfer (EET) but whose physiological relevance and mechanism of formation remain unknown.

Methods: Purified S. oneidensis OMVs were prepared by filtration and ultracentrifugation for comparative proteomics with cell-associated outer membrane proteins or for electrochemical measurements. Protein domains were predicted using HMMSCAN and CDD-search. OME formation and phenotype analyses were performed in situ by confocal and cryo-electron microscopy.

Results: The putative BAR domain-like protein BdpA was highly enriched in OMVs compared to cell-associated outer membranes. During OME biogenesis, WT S. oneidensis OMEs progress from elongated vesicle chains to narrow, tubule-like extensions while ΔBdpA OMEs remain as disordered vesicle chains. Purified OMVs from these strains are electrochemically active, with redox signals consistent with multiheme outer membrane cytochromes, supporting the role of OMEs in EET. Heterologous BdpA expression promotes OME formation in *Marinobacter atlanticus* and *Escherichia coli*, suggesting BdpA membrane sculpting activity is inducible and transferrable.

Summary/conclusion: The ability of BdpA to promote OME formation and maturation into tubules *in vivo* supports BdpA as a comparator for BAR domain protein activity in bacteria.

Funding: US DoD Synthetic Biology for Military Environments (SBME) Applied Research for the Advancement of Science and Technology Priorities (ARAP)

NSF Dimensions: DEB-1542527

US DOE: DE-FG02-13ER16415

PT06: EV Cancer Immunology
Chairs: Jason Webber; Koichi Furukawa
Location: Level 3, Hall A

15:30–16:30

PT06.01

Development of CD40L-modified small extracellular vesicles for the effective induction of anti-tumour immune response

Wen Liu[a], Yuki Takahashi[b], Masaki Morishita[c], Makiya Nishikawa[d] and Yoshinobu Takakura[b]

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Introduction: Tumour-derived small extracellular vesicles (sEVs) are anticipated to be a novel cancer vaccine because of their inherent encapsulation of tumour antigens. In this research, tumour-derived sEVs were modified with CD40 ligand (CD40L), which is a ligand for CD40 expressed on dendritic cells (DCs) and can activate DCs, in order to induce effective tumour antigen-specific immune response.

Methods: B16-BL6 murine melanoma cells were selected as sEVs-producing cells. Plasmid vector encoding a fusion protein of CD40L and lactadherin (LA), named as CD40L-LA, was constructed. B16-BL6 cells were transfected with the CD40L-LA-expressing plasmid vector and CD40L-modified sEVs (CD40L-sEVs) were collected from the culture medium of the transfected cells. The collected sEVs were characterized by using western blot, zeta sizer and transmission electron microscope (TEM). CD40L-sEVs labelled with PKH67 were added to DCs and the uptake of CD40L-sEVs was evaluated by flow cytometer. CD40L-sEVs were added to DCs and the cytokine release from the cells was measured by ELISA. Presentation of melanoma antigens contained in sEVs were evaluated by measuring cytokine release from melanoma antigen gp100-specific T cells, which were co-incubated with CD40L-sEVs added DCs. The concentrations of cytokines in the culture medium were determined using ELISA.

Results: The negatively charged sEVs with a diameter of approximately 100 nm were successfully modified with CD40L. CD40L-sEVs were more efficiently taken up by DCs than unmodified sEVs. DCs added with CD40L-sEVs produced more TNF-alpha and IL-12 than those added with unmodified sEVs. Moreover, CD40L-modification of sEVs improved the melanoma antigen presentation efficiency of DCs, which was demonstrated by increased IL-2 secretion from melanoma antigen gp100-specific T cells.

Summary/Conclusion: It was shown that CD40L-modified sEVs can be used to induce effective anti-tumour immune response.

PT06.02

Development of Interferon γ-loaded tumour cell-derived extracellular vesicles applicable to cancer vaccine therapy

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Introduction: Extracellular vesicles (EVs) contain various substances such as proteins and nucleic acids derived from their producing cells. As tumour cell-derived EV (TEV) contains tumour antigens, TEV is expected to be used as a cancer vaccine. However, since the immune activation ability of TEV is low, it is difficult to induce effective anti-tumour immunity by simple administration of TEV alone. Hence, in this study, we attempted to enhance the immune activation ability of TEV by loading Interferon (IFN)-γ.

Methods: A plasmid vector encoding a fusion protein of lactadherin that specifically bind to phosphatidylserine contained in EV membrane and mouse IFN-γ was prepared and the vector was transfected into a mouse melanoma cell line B16BL6 cells. Then, IFN-γ-loaded TEV (γ-TEV) was collected from the supernatant of the transfected cells by ultracentrifugation. IFN-γ loaded on the collected TEVs was detected by Western blotting and ELISA. IFN-γ biological activity of IFN-γ loaded on γ-TEV was evaluated by a reporter assay. In addition, γ-TEV was added to the mouse dendritic cell line, DC 2.4, and mRNA and protein expression levels of antigen presentation-related genes were analysed using RT-qPCR and FACS analysis. Finally, splenocytes of mice that had received intradermal administration of γ-TEV were collected and the amount of IFN-γ produced from the splenocytes incubated with B16BL6 antigens was measured.

Results: It was confirmed that IFN-γ was successfully loaded to TEV. In addition, the reporter assay confirmed that the biological activity of IFN-γ was retained

in γ-TEV. Addition of γ-TEV to DC 2.4 increased mRNA and protein expression of MHC class I and CD86 compared to TEV alone group, which suggests that immune activation ability of TEV was increased by loading IFN-γ. Furthermore, in the splenocytes assay, the amount of IFN-γ production was significantly increased in the γ-TEV administration group compared with the group administered with simple mixture of IFN-γ and unmodified TEV.

Summary/Conclusion: These results indicated that IFN-γ loading to TEV is an effective approach for cancer immunotherapy using TEV.

PT06.03

Apoptotic neuroblastoma derived extracellular vesicles can prime mesenchymal stem cells to decrease regulatory T cells differentiation

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Introduction: There are many ongoing studies investigating tumour derived extracellular vesicles (EVs). Yet in cancer patients receiving chemotherapy, a majority of the tumour are undergoing apoptosis and the difference between health cancer and dying cancers EVs are still unknown. Apoptotic tumour cells can secrete EVs containing different messages to the tumour microenvironment and effect the surrounding cells in a different way. Mesenchymal stem cell (MSC) is a heterogeneous multipotent stem cell found within the tumour microenvironment and can regulating the immune system. The aim of this study is to investigate the role of apoptotic EVs on mesenchymal stem cell immunomodulatory function in a tumour microenvironment.

Methods: EVs were obtained from both healthy SK-N-LP neuroblastoma cell line and those treated with the chemo drug cisplatin for 24 h. EVs were isolated from ultracentrifugation at 16,000 g for larger EVs and 100,000 g for smaller EVs. The characterization of the different populations of EVs was performed by western blot and nanoparticles tracking analysis. Neuroblastoma derived EVs were then co-cultured with immortalized human MSC (hTMSC) for 48 h. The immunomodulatory function of hTMSC was determined by their effect on T cells isolated from PBMC.

Results: T cells co-cultured with hTMSC have an increase in FoxP3 expression whereas hTMSC that has been primed with apoptotic EVs from neuroblastoma showed a significant decrease in FoxP3 expression. The DAMP molecule HMGB1 was found to be present in apoptotic EVs, whilst being absent in healthy neuroblastoma EVs.

Summary/Conclusion: Although MSCs are commonly known to have an immunosuppressive function, after the uptake of EVs derived from apoptotic neuroblastoma, MSC was able to switch to an immunostimulatory phenotype and decreasing Treg differentiation. Dying tumour cells may package danger signals and alarmins in their EVs thereby activating immune response in the tumour microenvironment.

Funding: The Edward & Yolanda Wong Research Fund

PT06.04

Chronic Lymphocytic Leukaemia-derived small extracellular vesicles: a potential strategy for immune escape

Ernesto Gargiulo[a], Sandrine Pierson[b], Bassam Janji[a], Jérôme Paggetti[a] and Etienne Moussay[a]

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Introduction: Chronic Lymphocytic Leukaemia (CLL) is the most common adult leukaemia and characterized by the accumulation of abnormal B lymphocytes. CLL cell survival and proliferation are highly dependent on interactions with the microenvironment. Thus, to identify effective strategies to impair tumour proliferation, it is essential to understand the communication between CLL and surrounding tissues.

Methods: To obtain a biological representation of small extracellular vesicles (small Evs) in the tumour microenvironment, we established a new protocol allowing us to isolate highly pure small Evs directly from the spleen of leukemic mice. Small Evs quality and sample purity were evaluated with qNano (TRPS principle), western blot and conventional bead-based flow cytometry. Next, we screened a wide range of immune checkpoint ligands on the surface of CLL-derived small Evs and corresponding receptors on the surface of T cells.

Results: We have succeeded in isolating small Evs generated by CLL cells *in vivo*. Our screen suggested the presence on immune checkpoint ligands directly anchored on tumour-derived small Evs. Furthermore, we identified a promising pair ligand-receptor potentially implicated in immune escape. Validation of candidates from the screen is currently being performed through FACS, iFACS and EM. These techniques will allow us to better define tumour-derived small Evs populations presenting different immune checkpoints and to visualize single small Evs with high resolution.

Summary/Conclusion: In this project, we aimed to isolate and characterize CLL-derived small Evs to

define their involvement in tumour development, with focus on the evaluation of their impact on CLL immune escape.

Altogether, this study will give insight into the specific immune and stromal cells involved in CLL development, with emphasis on their involvement in tumour-derived small Ev-mediated tumour immune escape.

Funding: This project is funded by the Fonds National de la Recherche (FNR) INTER/DFG/16/11509946/EV-RNA/Moussay.

Sandrine Pierson and Jérôme Paggetti are supported by the FNR INTER/DFG/16/11509946/EV-RNA/Moussay.

Ernesto Gargiulo is supported by the grant FNR Luxembourg PRIDE15/10675146/CANBIO.

PT06.05

Interaction via exosome miRNAs between myelodysplatic cell and normal Treg

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Introduction: Myelodysplastic Syndrome (MDS) is a clonalhematopoietic disease and develops leukaemia in some cases. Thus, MDS is a malignant hematopoietic disease and its prevalence ratio is increasing in Japan. Hematopoietic microenvironment such as bone marrow niche is a crucial factor for maintaining leukaemic stem cells. To understand mechanisms of interactions between leukaemic stem cells and microenvironment is important for the treatment of hematopoietic malignancies.

In this study, to develop the new therapies and diagnostic methods for MDS, we focused on the effect of exosomes released from MDS cells on peripheral T lymphocytes.

Methods: MDS cell line (MDS-L) was kindly provided by Kasawaki Medical University and normal peripheral blood mononuclear cells were obtained from healthy volunteer donors. Exosomes from MDS cells were purified by using miRCURY Exosome Cell/Urine/CSF Kit and labelled by PKH67. Extracted miRNAs were analysed by microarray method (Genopal, Mitsubishi Chemical, Japan). Cell surface antigens were analysed by FACS Aria II and fluorescence conjugated antibodies.

Results: miRNA-microarray analysis showed that nine miRNAs were abundant in exosomes from MDS cells and were not detected in MDS cells. Exosomes labelled with PKH67 dye were added to liquid culture of regulatory T cells (Treg) that were sorted from normal peripheral blood. The exosomes were detected in cytosol of Treg by fluorescent microscopy. Microarray analysis of miRNAs in Treg intaking MDS-exosomes showed that significant increases of 9 miRNAs in MDS-exosomes. The conditioned medium of MDS-exosomes treated Treg culture reduced the population of activated CD4 cells (CD38 positive cells was 39%; control 68%).

Summary/Conclusion: Our data suggested that exosomes from MDS cells affected the function of regulatory T cells via miRNA transfer. MDS exosomes may effect on immune cells to avoid the exclusion from cancer-immune system, and may be a target for the new therapies or diagnostic methods.

Funding: This work was supported in part by a grant from the Japan Society for the Promotion of Science (JSPS KAKENHI Grant Number: JP17K09020 and 17H07059).

PT06.06

Mechanism of antitumor immunity activation by 'artificial neoantigen'-presenting exosomes

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Introduction: Tumour-derived exosomes are known to have same antigens as the parent tumour cells, and were expected as cancer vaccines. However, treatment with those exosomes often failed to elicit antitumor immune responses, probably owing to a weak immunogenicity of the tumour-associated antigens (TAAs). TAAs can be divided into two categories, overexpressed self-antigens and tumour-specific mutated neoantigens. Recently, it was found that immunotherapy was effective only in patients whose tumours had neoantigens with high immunogenicity. However, most tumours do not have such efficient neoantigens. To overcome the disadvantage, we have developed an "artificial neoantigen strategy". Previously, we prepared the exosomes expressing strong bacterial antigen, as an "artificial neoantigen" by transformation of the original cultured cells with a gene of the Mycobacterium tuberculosis antigen, early secretory antigenic target-6 (ESAT-6). Injection of the exosomes induced significant antitumor activity in tumour bearing mice. Here we investigated the mechanism of antitumor immunity activation by the "artificial neoantigen"-presenting exosomes.

Methods: Mouse B16 melanoma cells were transfected with ESAT-6, and secreted ESAT-6 antigen-presenting exosomes (ESAT-Ex) were isolated. Cultured mouse DCs were treated with the exosomes and expression of costimulatory molecules and cytokines was assessed. Antitumor activity of the ESAT-Ex-stimulated DCs was evaluated in tumour bearing mice. In vivo immune activating ability of ESAT-Ex was investigated by co-incubation of the lymphocytes taken from the ESAT-Ex-treated mice with B16 tumour cells. Immuno-stimulation by the fibroblasts-secreted ESAT-Ex was also studied.

Results: The DCs stimulated with ESAT-Ex showed enhanced CD80 and CD86 presentation, and exhibited significantly improved antitumor activity in tumour-bearing mice. When the lymphocytes harvested from the mice injected with ESAT-Ex were co-incubated with B16 cells, they intensely accumulated around the tumour cells, and secreted higher level of IFN-gamma. Increased uptake of thymidine was also observed.

Summary/Conclusion: "Artificial neoantigen"-presenting exosomes effectively stimulated DCs and evoked antitumor immunity. They are expected as novel cancer vaccines.

Funding: This work was supported by JSPS KAKENHI Grant Number 16K01394.

PT06.07

Outer membrane vesicles of *Tannerella forsythia* induce inflammatory response in periodontal tissue cells

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Introduction: *Tannerella forsythia*, a Gram-negative oral bacterium, is one of the major periodontal pathogens which can cause inflammatory responses. Inflammasome is crucial for host defence against pathogens, but excessive inflammasome activation can lead to tissue damage. Outer membrane vesicles (OMVs) are derived from the cell envelope of Gram-negative bacteria. OMVs can contain DNA, RNA, lipopolysaccharide, proteins, toxins and peptidoglycan. *T. forsythia* induces maturation of IL-1α/IL-1β and cell death via activation of caspase-1/4 in THP-1 macrophages and human gingival fibroblasts (HGFs). The aim of this study was to investigate whether *T. forsythia* OMVs are involved in inflammasome activation which may contribute to periodontitis, a chronic inflammatory disease.

Methods: *T. forsythia* OMVs were isolated using Exo-bacteria OMVs isolation kit. THP-1 macrophages differentiated with PMA and HGFs were treated with *T. forsythia* and OMVs at various does. The expression of caspase-1/4 and pro-inflammatory cytokines was determined by immunoblotting and ELISA, respectively. Cell death was measured by LDH cytotoxicity assay kit.

Results: *T. forsythia* OMVs activated caspase-1 and −4, resulting in increased IL-1α and IL-1β release and inflammatory cell death. The OMVs also induced the expression of IL-6 and IL-8.

Summary/Conclusion: The results indicated that *T. forsythia* OMVs may play an important role in inflammatory response in *T. forsythia*-infected cells.

Funding: This work was supported by National Research Foundation of Korea grants (No. NRF-2018R1A5A 2024418 and NRF-2018R1A2A2A05018558).

PT06.09

Specific decrease of CD19+extracellular vesicles enhances post-chemotherapeutic CD8+T cell responses

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Introduction: Chemotherapy has long been related with induction of systemic immunosuppression. Systemic immunosuppression greatly affects chemotherapeutic antitumor effect. Therefore, amelioration of systemic immunosuppression following chemotherapy is necessary to improve post-chemotherapeutic antitumour immunity.

Methods: CD19+EVs were isolated and identified by EM, NTA, FACS and western blotting. CD19+EVs functions on degrading ATP and their immunosuppressive functions were assessed *in vitro* and *in vivo*. The effects of CD19+EVs on antitumour effect of chemotherapy were detected by transfer of exogenous EVs into tumour mice or in Rab27a or Hif1a conditional knockout tumour mice. The effects of CD19+EVs on antitumor effect of chemotherapy were also evaluated in humanized NSG mice by knocking down Rab27a with inactivated EBVs loading with Rab27a siRNA.

Results: CD19+extracellular vesicles (EVs) from B cells hydrolyse ATP from chemotherapy-treated tumour cells into adenosine by CD39 and CD73, resulting in impaired CD8+T cell responses. Serum CD19+EVs increase in tumour mice and patients. Patients with fewer serum CD19+EVs have a better prognosis following chemotherapy. Up-regulated HIF-1α promotes B cells releasing CD19+EVs by inducing Rab27a mRNA transcription. Rab27a or HIF-1α deficiency in B cells inhibits CD19 +EV production and strikingly improves

chemotherapeutic antitumor effect. Knockdown of Rab27a in B cells by inactivated Epstein-Barr viruses carrying Rab27a siRNA greatly improves chemotherapeutic efficacy in humanized NSG mice.

Summary/Conclusion: Our findings unravel a mechanism underlying systematic immunosuppression after chemotherapy. Combination of chemotherapy and iEBVs/Rab27a siRNA holds high potential for cancer treatment.

Funding: This work was supported by grants from the National Key Basic Research Program of China (2015CB943301), the National Key R&D Program of China (2016YFA0501800), and the National Natural Science Foundation of China (31770951 and 31670877).

PT07: EVs in Acute and Chronic Inflammatory Disorders
Chairs: Eric Boilard; Aleksandra Gasecka
Location: Level 3, Hall A

15:30–16:30

PT07.01

Circulating microvesicles as potential biomarkers of Acute Respiratory Distress Syndrome in Sepsis

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Introduction: Acute respiratory distress syndrome (ARDS) is a clinical condition of sudden respiratory failure in critically ill patients. ARDS-related mortality rate is higher when is associated with Sepsis (>50%). Recently, we screened 754 miRNAs and discovered a different cargo transported by circulating extracellular vesicles (EVs) and exosomes from patients with sepsis, remarkably in those who progressed to death. The early sequence of events of respiratory failure after the onset of sepsis are still unknown. Our hypothesis is that lung should signal through EVs that it is being affected by SIR.
Methods: Blood samples were obtained from septic patients with (n = 8) and without ARDS (n = 5) at 24 h of intensive care unit (ICU) admission and 3 days later at Sirio-Libanes Hospital. Pulmonary originated sepsis was not considered. Eight patients under mechanical ventilation (MV) without pulmonary disease and 12 healthy volunteers were used as controls. Plasma was 0.22 µM filtered, EVs were isolated by ultracentrifugation and analysed by nanoparticle tracking analysis. Based on our previous data, 48 miRNAs were measured by Taqman Low Density PCR array and normalized by RNU6.
Results: The main population of EVs peaked at size of 155–165 nm with no difference in the mean concentration between groups. Patients with sepsis + ARDS showed a significant decrease in plasma EVs 3 days after ICU stay (234 to 137 x 10e8/mL, p = 0.0175). Compared to healthy donors, sepsis promotes an even significant alteration of EVs-miRNAs when it is associated with ARDS. Comparing all samples from patients with sepsis + ARDS to sepsis only, nine miRNAs are transported in smaller amounts: miR-766 (−35.7, p = 0.002), miR-127 (−23.8, p = 0.001), miR-340 (−13.5, p = 0.006), miR-29b (−12.8, p = 0.001), miR-744 (−7.1, p = 0.05), miR-618 (−4.0, p = 0.02), miR-598 (−3.8, p = 0.035), miR-1260 (−2.5, p = 0.035); and miR-885-5p is expressed at higher levels (9.5; p = 0.028). In paired samples, the set of altered miRNAs is generally different (p < 0.05) between sepsis + ARDS (miR-148a, −193a-5p, 199a-3p, −222, −25, −340, 744) or sepsis only (miR-1183, −1267, −1290, −17, −192, −199a-3p, −25, −485-3p, −518d, −720).
Summary/Conclusion: Circulating EV-miRNAs cargo could be potential biomarkers of lung inflammation during sepsis in patients who will require MV.
Funding: FAPESP.

PT07.02

Innate/ inflammatory cross talk between macrophages (Mps) and RPE cells are mediated by exosomes secreted by RPE cells: Proposal of new trait for the pathogenesis of age-related macular degeneration (AMD)

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Introduction: The pathogenesis of AMD is aggravated by chronic inflammation. Intact RPE down-regulates the production of TNF-alpha by choroid-infiltrating Mps, whereas degenerated RPE by oxidative stress were devoid of this regulatory function. Subsequently, locally produced TNF-alpha induces the production of some pro-inflammatory cytokines and angiogenic factor VEGF by RPE (Yamawaki et al., 2016). This implies that innate/inflammatory cross talk between Mps and RPE may be the indispensable trait for AMD pathogenesis.

The purpose of this study is to elucidate the signal that causes up-regulated TNF-alpha production in congenital / inflammatory crosstalk between Mps and RPE.
Methods: Mps cell line RAW 264.7(RAW) was co-cultured with primary RPE taken from C57BL/6 mice. Some cytokines in the culture supernatants (CSs) were quantified by ELISA. The expression profiles of complement-associated genes, TNF-alpha, and

angiogenesis-associated genes (VEGF & PEDF) were analysed by qRT-PCR. For the preparation of exosomes (Exo), CSs were harvested after co-cultures of RAW with primary RPE, then Exo in each CSs were purified by either EVsecondTM or ultracentrifugation. The incorporation of the Exo either into RPE or RAW was histologically quantified using Qdot 655 streptavidin conjugated biotinylated Exo.

Results: Elevated levels of CD63 positive Exo in co-cultures were detected by western blot or FACS analysis. The produced Exo in co-culture CSs were incorporated solely into RAW, but not into RPE. The semi-purified Exo, but not the Exo depleted residual CSs enhanced the secretion of MCP-1 and IL-6 in co-culture of Mps and RPE, while the enhancement of VEGF are similarly detected by the Exo deprived residual CSs. Most remarkable elevation was observed in TNF-alpha production by RAW in a dose-dependent manner even in the absence of RPE. The down-regulated TNF-production by RAW in the presence of RPE was not reconstituted by the addition of Exo even in the co-culture.

Summary/Conclusion: Exosome displays a critical role in the triggering of vicious inflammatory cytokines cycle through the elevation of TNF- production by Mps.

Currently, in order to construct an experimental system closer to the pathology of AMD, we are studying a co-culture system using human Mps and human iPS-derived RPE.

PT07.03

Epithelial exosomes regulated by phosphatase Shp2 promote macrophage activation

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Introduction: Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are life-threatening diseases that are associated with high mortality rates due to treatment limitations. Increasing researches suggest exosomes play an important role in pathogenesis, diagnosis and treatment of ALI. However, it's not clear how exosomes are formed, secreted, transferred during ALI. Phosphorylation of signalling proteins are reported to control exosome biogenesis (e.g. syntenin phosphorylation promotes exosome formation). Shp2 is a widely expressed cytoplasmic phosphatase which can regulate signalling pathway by its dephosphorylation function. Here we reveal that Shp2 inhibits the biogenesis of epithelial exosomes which have proinflammatory effects on macrophages during ALI. It's uncovered in our study that Shp2 is a protective factor of ALI by inhibiting release of proinflammatory epithelial exosomes.

Methods: Exosomes were isolated by differential ultracentrifugation and filtration, and they were characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and western blot (e.g. CD9, CD63, CD81, ALIX, TSG101). *In vitro* transwell system for exosome transfer model indicated the direction of exosome transfer. Nanoscale flow cytometry (CytoFLEX) was used for detecting exosome subpopulation.

Results: Exosomes were increased in Bronchoalveolar Lavage Fluid (BALF) of LPS-induced ALI murine model. *In vitro* transwell system revealed that exosomes were transferred from epithelial cells to macrophages in inflammation environment. Shp2 was revealed to inhibit the biogenesis of epithelial exosomes without changing their size and subpopulation. Adaptor protein Gab2, which can bind Shp2, was found to interact with Syntenin. It suggests that with the help of adaptor Gab2, Shp2 was involved in dephosphorylating syntenin whose phosphorylation can facilitate exosome biogenesis. Shp2-disruption derived epithelial exosomes promoted macrophage inflammation, thus aggravating ALI.

Summary/Conclusion: Our study shows that phosphatase Shp2 inhibits proinflammatory epithelial exosome release, which can promote M1-macrophage polarization. It offers a potential target for ALI mechanism study and treatment.

PT07.04

Detection of CD11b-expressing exosomes in plasma of mice with sepsis

Yasunori Fujita, Kyojiro Kawakami and Masafumi Ito

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Introduction: Cells communicate with each other through extracellular vesicles including exosomes, which contain host cell-derived molecules such as proteins, lipids and nucleic acids. Secreted exosomes migrate not only to neighbouring cells but also to distant organs. Monocyte and macrophage have been reported to secret exosomes that modulate immune responses. However, the characteristics of monocyte/ macrophage-derived exosomes in blood during

systemic immune response remain largely unknown. In this study, we characterized exosomes released from monocyte/macrophage-like cells and determined the temporal change in monocyte/macrophage-derived exosomes in plasma of mice with sepsis.
Methods: Exosomes collected by ultracentrifugation from the conditioned medium of lipopolysaccharide (LPS)-stimulated murine monocyte/macrophage-like RAW264.7 cells were subjected to quantitative proteomic analysis using iTRAQ labelling and LC-MALDI-TOF/TOF. Plasma exosomes isolated from LPS-injected mice were analysed by Western blot analysis. CD11b-expressing exosomes in plasma were measured by sandwich ELISA. Plasma TNF-α level was determined by ELISA.
Results: Proteomic analysis showed that monocyte/macrophage marker proteins such as CD11b, CD14 and F4/80 were detected in exosomes from RAW264.7 cells. Glucose metabolism-related proteins including GLUT1, PKM2 and GAPDH increased in exosomes from LPS-stimulated cells compared with those from non-treated cells. Western blot analysis demonstrated that GLUT1 and CD11b were significantly increased in plasma exosomes from LPS-injected mice. After LPS stimulation, TNF-α transiently increased, whereas CD11b-expressing exosomes increased and remained high in plasma of mice with sepsis.
Summary/Conclusion: We characterized monocyte/macrophage-derived exosomes in plasma of mice with sepsis and developed a sandwich ELISA for detection of CD11b-expressing exosomes in plasma, which could be a novel marker for systemic immune response as well as sepsis.
Funding: JSPS KAKENHI Grant Number JP17K01888.

PT07.05

Systemic inflammatory activity and proteome analysis of extracellular vesicles from faeces

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Introduction: Substantial quantities of bacteria reside in the gastrointestinal tract. Severe inflammatory responses are induced when the bacteria went through the peritoneum from the gastrointestinal tract. In this study, extracellular vesicles isolated from faeces (fEVs) were assessed to see whether they could mediate inflammatory responses. In addition, proteomic compositions of fEVs were further investigated.
Methods: The faeces of wild-type mice were utilized to isolate fEVs. The fEVs were characterized with transmission electron microscopy, dynamic light scattering, ELISA, and Western blot. The fEVs were intraperitoneally administered into the mice, and the number of infiltrated cells as well as the concentrations of TNF-α and IL-6 were measured from the peritoneal lavage fluid, serum, and bronchoalveolar lavage fluids. Proteomic analyses on the fEVs were conducted by the combination of one-dimensional SDS-PAGE and LC-MS/MS.
Results: Significant amounts of fEVs were isolated from mouse faeces, and the fEVs were derived from bacteria and host cells. Upon intraperitoneal administration, the fEVs mediated peritoneal, systemic, and pulmonary inflammation by increasing the numbers of infiltrated immune cells and the pro-inflammatory cytokines such as TNF-α and IL-6 in the peritoneal lavage fluid, serum, and bronchoalveolar lavage fluid. Proteomic analyses on the fEVs identified a total of 295 proteins, comprising 222 bacterial proteins and 73 murine proteins.
Summary/Conclusion: The fEVs derived from bacterial and host cells could mediate local and systemic inflammation, and composed of bacterial and host proteins. These results shed lights on the roles of commensal bacterial EVs in the pathogenesis of inflammatory diseases.
Funding: National Research Foundation of Korea (NRF) Herman Krefting Foundation for Allergy and Asthma Research, Lundberg Foundation

PT07.06

Opioid-mediated release of astrocytic EV miR-23 induces pericyte migration and blood-brain barrier breach

Shilpa Buch, Ke Liao, Fang Niu and Guoku Hu

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Introduction: Pericytes are important constituents of the cerebrovascular unit and play a key role in maintaining the integrity of the blood-brain barrier. It is well recognized that drugs of abuse such as opioids can result in breach of the BBB, ultimately leading to enhanced monocyte transmigration and ensuing neuroinflammation. Mechanism(s) by which pericytes contribute to morphine-mediated neuroinflammation, however, remains less understood.
Methods: EVs were isolated from morphine-stimulated mouse/human primary astrocytes using the standard

differential ultracentrifugation method and characterized by transmission electron microscopy, NanoSight & western blot analyses. Among the various miRs dysregulated in morphine-stimulated astrocyte EV cargo, miR-23 was found to be upregulated by real-time PCR. Confocal microscopy identified uptake of astrocytic EVs by pericytes. Functional assessment of astrocytic EV uptake by pericytes involved cell migration using Boyden chamber and wound healing assays. Additionally, an *in vitro* 3D model comprising of pericytes and human endothelial cells was also used to assess astrocyte EV-mediated migration of pericytes in presence of morphine.

Results: Exposure of astrocytes to morphine induced the expression and secretion of miR-23 in the EVs, which, upon uptake by the pericytes resulted in their migration. Additionally, in the pericytes that had taken up morphine stimulated astrocyte EVs, there was downregulation of phosphatase and tensin homologue (PTEN), a target of miR-23.

Summary/Conclusion: Our findings indicate that morphine-mediated dysregulation of miRNA expression in the CNS involves astrocyte-pericyte communication via the extracellular vesicles, leading, in turn, to loss of pericyte coverage at the BBB.

Funding: This work was supported by grants DA040397, MH112848 (S.B.) and DA042704, DA046831 (G.H.) from the National Institutes of Health. The support by Nebraska Center for Substance Abuse Research is acknowledged.

PT07.07=OWP2.15

Diagnostic microRNA biomarkers from circulating extracellular vesicles for early detection of pneumonia and severe secondary complications

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Introduction: Pneumonia remains one of the most deadly communicable diseases, causing three million deaths worldwide in 2016. Extracellular vesicles (EVs) are pivotal during signal transfer in the pathogenesis of inflammatory lung diseases. Since identifying pneumonia is particularly challenging in high risk groups (e.g. the elderly or infants), which often present with atypical symptoms and are at high risk for secondary complications such as sepsis or acute respiratory distress syndrome (ARDS), new approaches for early diagnosis are required. In this study we identified EV microRNAs (miRNAs) as potential biomarkers for inflammatory changes of the pulmonary tissue.

Methods: Our study included 13 patients with community-acquired pneumonia, 14 ARDS patients, 22 patients with sepsis and 31 healthy controls. After precipitating EVs from 1 ml serum, total RNA was extracted. Subsequent to library preparation and small RNA-Seq, differential gene expression analysis was performed using DESeq2. Data were filtered by mean miRNA expression of $\geq$ 50 reads, minimum twofold up or down regulation and adjusted p-value $\leq$ 0.05.

Results: The mean relative miRNA frequency varied slightly between the different groups and was highest in volunteers. Short sequences (< 16 nucleotides), probably degradation products from longer coding and non-coding RNA species, were predominantly detected in patients. Based on unsupervised clustering, patients could be distinctly separated from healthy individuals. Although 21 miRNAs were significantly regulated in all patient groups compared to healthy controls, different disorders showed unique miRNA expression profiles. Distinct miRNA subsets were identified, which are applicable to indicate disease progression from limited inflammation present in pneumonia to severe inflammatory changes as seen in ARDS and sepsis.

Summary/conclusion: This study shows that EV miRNA biomarkers have potential for diagnosis of pneumonia and to indicate disease progression towards severe lung injury. Our findings are of clinical relevance, as the timely diagnosis of pneumonia can be challenging, and secondary complications such as ARDS and sepsis might be prevented by early intervention and treatment.

Funding: This study was supported by the German Federal Ministry for Economic Affairs and Energy under the program “Zentrales Innovationsprogramm Mittelstand”.

PT08: EVs in Metabolism and Metabolic Diseases
Chairs: Sophie Rome; Alena Ivanova
Location: Level 3, Hall A

15:30–16:30

PT08.01

Elevated levels of platelet and endothelial extracellular vesicles in type 1 diabetes, a cohort study of 236 patients

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Introduction: We have recently presented data on increased levels of circulating extracellular vesicles (EVs) in patients with type 1 diabetes. In the same cohort, we have now analysed subpopulations of platelet- and endothelial EVs in relation to diabetic microangiopathy and sex.

Methods: Two hundred and thirty-six patients (107 women) and 100 healthy controls matched for age, sex and body mass index (BMI) gave written informed consent to the study. Plasma platelet EV (PEV) and endothelial EV (EEV) levels were assessed using flow cytometry with labelled antibodies directed against platelet (CD42a and CD61) and endothelial specific (CD144 and CD62E) antigens. EV expression of procoagulant phosphatidylserine (PS) and tissue factor (TF) were assessed using lactadherin (lac) and CD142 antibody. The study was approved by the local ethics committee.

Results: PEV and EEV levels with or without expression of procoagulant PS and TF were statistically higher among patients than in controls ($p < 0.05$ for all). The patients had about 50% higher PEV levels and up to a 50-fold increase in EEV levels compared to controls. No statistically significant differences were found between PEV or EEV levels in patients with or without clinical microangiopathy. Healthy women had lower PS+, PS− and total PEV levels compared to healthy men ($p < 0.05$ for all), whereas no differences between sex were found in the patients. PEV and EEV levels in patients did not correlate with glycaemic control (HbA1c), BMI, blood pressure, blood lipids or diabetes duration.

Summary/Conclusion: Elevated levels of PEVs and EEVs found in patients with type 1 diabetes are unrelated to clinical microangiopathy, indicating that type 1 diabetes is a procoagulant state per se. Comparison of PEV levels between the sexes showed a more favourable phenotype in healthy women compared with healthy men, while no sex differences were found among patients. This could be linked to the loss of female protection against cardiovascular disease in type 1 diabetes.

Funding: Berth von Kantzow Foundation, Swedish Diabetes Foundation, Wallenius Foundation, Swedish Heart-Lung Foundation, Foundation of Women and Health

PT08.02

Role of extracellular vesicles in the regulation of inflammation and metabolism in obesity

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Introduction: The worldwide prevalence of obesity has reached pandemic proportions. Obesity has strong inflammatory underpinnings, which are associated with the development of type 2 diabetes (T2D) and non-alcoholic steatohepatitis (NASH). However, the mechanisms by which obesity provokes aberrant inflammation have yet to be clearly defined. Extracellular vesicles (EVs), including exosomes and microvesicles, are a novel mode of tissue-to-tissue communication. Recent studies indicate that EVs are involved in many pathophysiological events including inflammatory responses and metabolic dysfunctions. We hypothesize that EVs play critical roles in the induction of obesity-associated aberrant inflammation and the development of metabolic diseases.

Methods: To investigate the role of EVs in the pathogenesis of obesity, we have taken systematical approaches including novel computational methods, analyses of EVs collected from human obese patients undergoing bariatric surgery, utilization of novel

mouse models monitoring cell type-specific EVs, and cellular-based EV functional assays.

Results: Using novel computational methods, we have identified strong associations with EV-related genes in metabolic syndrome associated with T2D. Our analyses of EVs from adolescent obese patients undergoing bariatric surgery have shown that serum EV concentration is inversely correlated to metabolic improvements in glucose metabolism and inflammation post-surgery, with unique EVs' extracellular RNA (exRNA) profiles. Further, our newly established mouse models monitoring specific cell type-derived EVs *in vivo* indicates that in obesity, EVs from metabolic tissues behave like a pathogen and induce inflammation.

Summary/Conclusion: While the research of EVs has attracted much attention, therapeutic targeting and significance of EVs in metabolic diseases are still a controversial area of research. By utilizing our novel mouse models coupled with access to human samples, our systematical approaches allow to propose novel mechanisms by which pathologic EVs induce aberrant inflammation and deteriorate metabolism in obesity.

PT08.03

Characterization of exosomal proteins derived from contracting skeletal muscle as potential mediators of beneficial metabolic effects of exercise

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Introduction: Exercise training improves glucose metabolism and insulin sensitivity supporting the concept that lifestyle modification is useful for patients to prevent and treat type 2 diabetes. Skeletal muscle also releases circulating factors following exercise affecting metabolism of other organs probably involving the release of exosomes. However, little is known about muscle-specific release of exosomes and the differential protein content of exosomes during exercise. Thus, we aimed to identify exosomal proteins regulated by exercise *in vitro* and *in vivo* and to discover pathways regulated by exosomal cargo.

Methods: Exosomes from human skeletal muscle cells (hSkMC), contracted by electrical pulse stimulation (EPS), were isolated by differential ultracentrifugation/ultrafiltration. Exosomes were also isolated by size exclusion chromatography from plasma of patients with and without type 2 diabetes on high intensity interval training (HIIT). In order to allow reliable label-free quantification of the very limited muscle exosomal material, we performed proteomic profiling using data independent acquisition (DIA) on an OrbitrapTM Fusion Lumos instrument. Spectronaut TM Pulsar software was used to integrate spectral libraries and perform quantitative proteomic profiling of exosomes derived from different human primary cells as well as human serum and plasma.

Results: EPS stimulated the release of exosomes from hSkMC and regulated the release of 408 exosomal proteins. Ingenuity pathway analysis (IPA) revealed significant regulation of, e.g. integrin, vascular endothelial growth factor, Liver X receptor/Retinoid X receptor and PI 3-kinase/Akt signalling by these proteins. HIIT regulated the amount of 62 exosomal plasma proteins *in vivo*, of which nine candidates were also similarly regulated by EPS *in vitro*. IPA links these exosomal proteins to pathways involved in metabolic diseases and lipid metabolism.

Summary/Conclusion: We identified various exercise-regulated exosomal proteins with predicted metabolic effects. Further analysis of these novel tissue-specific candidates will help to better understand systemic metabolic effects of exercise.

PT08.04

Transcriptome and proteome of extracellular vesicles derived from cellular targets of diabetic kidney disease

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Introduction: Kidney disease (DKD) is common, costly and the most feared complication of long standing diabetes. Its root causes remain unknown. Interestingly the characteristic changes in circulating glucose levels in this disease appear to alter the signature of extracellular vesicles as found in the urine. Here we wanted to explore the EV secretion pattern by key DKD target cells in the glomerulus (podocytes, endothelial and mesangial cells) and proximal tubular cells by harvesting the entire EV repertoire using Hydrostatic Filtration Dialysis (HFD).

Methods: We used cell culture media from podocytes, proximal tubule, mesangial and glomerular endothelial cells in four conditions: (1) Insulin sensitive, (2) Insulin resistant, (3) Insulin receptor transfected and insulin sensitive, (4) Insulin receptor transfected and

insulin resistant. EVs were isolated from 50 mL of cell culture media, respectively, by HFD. Quality of the EV yield was verified with negative staining Electron Microscopy (EM) and Western blotting (WB). Vesicle concentration was determined by Nanoparticle Tracking Analysis (NTA). Isolated RNAs were profiled with Bioanalyzer Pico kit and subjected to miRNAseq and RNAseq. EV proteins were analysed using tandem mass tag labelling.

Results: The isolated EVs appeared typical at EM and were positive for the EV-marker TSG101 in WB. RNA quantity and quality proved appropriate for both miRNA and RNAseq. Different treatments affected characteristically the vesiculation from the investigated target cells of diabetes. Ninety-six EV miRNAs could characteristically discriminate between the cell types and special treatments studied. Some EV miRNAs showed treatment effects and the analysis of their target genes using KEGG disease database showed a clear link to kidney diseases. Integrated miRNA-mRNA and protein analysis was also performed.

Summary/Conclusion: EV analysis provides a novel approach to reveal valuable pathophysiology, pathway and signalling information of cultured disease target cells. Changes in EV miRNAs, mRNA and proteomics may thus give valuable insight into mechanisms and targets to insulin resistance on DKD target cells.

Funding: BEAt-DKD, Paulo Foundation and Novo Nordisk Foundation.

PT08.05

Effects of an acute exercise on circulating extracellular vesicles: tissue-, gender- and BMI-related differences

Jacopo Mariani[a], Antonello Rigamonti[a], Silvano Cella[a], Alessandra De Col[b], Federica Rota[c], Sabrina Cicolini[b], Gabriella Tringali[b], Roberta De Micheli[b], Valentina Bollati[a], Sartorio Alesandro[b] and Mario Barilani[d]

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Introduction: Exercise is recognized to evoke multi-systemic adaptations that, particularly in obese subjects, reduce body weight, improve gluco-metabolic control, counteract sarcopenia and lower the risk of cardiometabolic diseases. Understanding the molecular mechanisms of exercise-induced benefits is of great interest due to the ensuing therapeutic implications against obesity.

Aims: To characterize extracellular vesicles (EVs) in obese (F/M = 8/8; age = 21.0 ± 8.5 years, BMI = 37.9 ± 6.0 kg/m^2) and normal-weight (F/M = 4/4; age = 25.1 ± 8.2 years, BMI = 20.9 ± 1.5 kg/m^2) subjects who underwent a moderate-intensity (60% VO_{2max} for 30 min or until exhaustion) exercise on a treadmill

Methods: Blood samples were drawn before, at the end and during post-exercise recovery period (3 and 24 h). EVs were analysed by Nanosight and flow cytometry after labelling with the following markers: CD14+ (monocyte), CD61+ (platelet), CD62E+ (activated endothelium), CD105+ (resting endothelium), HERVW+ (human endogenous retrovirus W), SCG+ (muscle) and FABP+ (adipose tissue).

Results: After exercise, 100–200 nm EVs significantly decreased ($p < 0.01$). There was a significantly higher post-exercise release of these EVs in normal-weight than obese subjects ($p = 0.025$). Considering the 30–130 nm size range, there was a significant lower release of EVs in females than males ($p < 0.01$). After exercise, the 130–700 nm EVs significantly decreased ($p = 0.016$). There was a higher release of these EVs in females than males ($p = 0.05$). After exercise, CD61+ EVs significantly decreased in all subjects ($p = 0.02$). SCG+ EVs were increased after exercise ($p = 0.06$). There were no significant associations of other biomarkers

Summary/Conclusion: An acute exercise induces changes in the release of plasma EVs, which are tissue-, gender- and BMI-specific, suggesting that the exercise-related benefits might depend upon a complex interaction of tissue, endocrine and metabolic factors

Funding: The study was supported by Progetti di Ricerca Corrente, Istituto Auxologico Italiano, IRCCS, Milan, Italy.

PT08.06

Profiling of extracellular vesicles from human hepatic stellate cells

Cristina Zivko[a], Gina Valentino[a], Kathrin Fuhrmann[b], Gregor Fuhrmann[c] and Paola Luciani[a]

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Introduction: The role of extracellular vesicles (EVs) in intercellular communication makes them particularly interesting in the research of many pathologies, especially those diagnosed invasively. In this study we evaluated differences in the EVs shed from human hepatic stellate cells (HSCs) in their different phenotypical states, since the activation of HSCs plays a

pivotal role in the progression of hepatic fibrosis, for which liver biopsy is still the diagnostic gold standard.
Methods: Protocols were optimized as to induce the different activation states in the cells; untreated HSCs were compared to their activated and quiescent counterparts. EVs were isolated from conditioned cell culture medium (CCM) after differential centrifugation followed by an ultracentrifugation (UC) step, and they were purified by size exclusion chromatography (SEC). The purification was evaluated by protein content determination by bicinchoninic acid (BCA) assay. The concentration and size distribution profiles of EVs in the SEC fractions were determined by nanoparticle tracking analysis (NTA). EV-morphology was observed by scanning and cryogenic transmission electron microscopy (SEM and cryo-TEM).
Results: Purification by SEC resulted in a distinct resolution between EVs and protein aggregates as determined by BCA assay. Protein content associated with EVs was only detectable in the SEC-fractions with the highest EV-yields and was comparable in all groups. Differently treated HSCs yielded EVs in similar amounts and size distributions. Quantile subtraction of the distribution curve obtained from untreated cells shows that activated HSCs produce smaller EVs (80–150 nm) more prominently than quiescent cells. SEM imaging confirmed the polydispersity in the samples.
Summary/Conclusion: EVs originating from differently treated HSCs were isolated and characterized in terms of yield, size, morphology and general protein content. Our results were consistently indicative of differences in EV populations originating from the same cells in either healthy or diseased state (quiescent or activated respectively), thus creating an important basis for the potential non-invasive detection of liver diseases.
Funding: The Phospholipids Research Center is gratefully acknowledged for its support to the project and Lipoid GmbH for the endowment to the University of Jena.

PT08.07

Role of exosomal miR-15a in diabetic retinopathy

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Introduction: Diabetic retinopathy is a debilitating complication of diabetes mellitus which results in irreversible blindness. Currently treatment is only initiated when significant damage from diabetic retinopathy has occurred. Early signs of damage typically remain unnoticed until it has reached advanced stages of disease. Identifying early biomarkers of disease will allow clinicians to detect the progression of disease before the onset of complications. Circulating microRNA contained in extracellular vesicles such as exosomes are potential early biomarkers and can be targeted to prevent diabetes from progressing. The aim of our project is to validate and determine the role of miR-15a as a potential early biomarker in diabetic retinopathy.
Methods: This project was approved by the University of Malaya Medical Centre (UMMC) Medical Research Ethical Committee. A total of about 100 subjects (controls and patients with Type 2 DM) was recruited from UMMC, Kuala Lumpur. All subjects underwent complete eye examination and graded for diabetic retinopathy. Clinical information collected included HbA1C, renal function testing, hypertension and smoking. Extracellular vesicle (EV) isolation was performed using differential ultracentrifugation and quantified.
Results: In this study, we analysed miR-15a concentrations in plasma and exosomal-enriched fractions using droplet digital and real-time PCR. There was no difference in microRNA levels in plasma observed. However, there was a significant increase in exosomal concentration (average diameter <130nM) in patients with diabetic retinopathy compared to controls ($p < 0.05$). There was also an increasing trend of miR-15a level among diabetic patients compared to controls.
Summary/Conclusion: The findings from this study corroborated with our previous findings of increase in miR-15a levels in diabetes prior to the onset of retinopathy compared to controls. This suggests that miR-15a is involved in the early development of diabetic microvascular complications and may be a potential biomarker for early complications of diabetes.
Funding: 1. Bayer Global Ophthalmology Award Program Grant. 2. University of Malaya Special Research Fund (BKS056-2017). 3. BioRad Institutional Funding (Materials and Lab consumables). 4. Fulbright Visiting Research Scholar Grant

PT08.08

The effects of outer membrane vesicles delivered from *Porphyromonas gingivalis* on hepatic glucose metabolisms

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and Pharmaceutical Sciences, Tokushima, Japan; [d]Departments of Oral Healthcare Promotion, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan

Introduction: The outer membrane vesicles (OMVs) of *Porphyromaons gingivalis (Pg)*, a gram-negative bacteria known as a major pathogen of periodontal diseases, include its virulence factors and regulate the aetiology of periodontal diseases by affecting microbial environment and the host cells in the oral cavity. However, it is unknown whether *Pg* OMVs in oral cavity could translocate to distant organ and affect the systemic diseases, whereas periodontal diseases are well known to influence the develop of diabetes mellitus. To elucidate the mechanisms by which periodontal diseases progress diabetes mellitus, we identified *Pg* OMV cargo proteins and verified its effects on the insulin signalling *in vitro*. We also analysed the translocation of *Pg* OMVs to the organ, and assessed the changes of hepatic glucose matabolisms in *Pg* OMV-treated mice.

Methods: We identified the OMV cargo proteins by LC-MS/MS analyses. The effects of *Pg* OMV on the insulin signalling in HepG2 cells is analysed by western blot. The organ distribution of OMV was analysed by IVIS sectrum after injecting intraperitoneally Cy7-labelled *Pg* OMV. We also estimated the insulin sensitivity using glucose tolerance test (GTT), insulin tolerance test (ITT) in mice treated with *Pg* OMV for 3 weeks.

Results: *Pg* selectively sorted its specific proteases such as arginine-specific gingipain (Rgp) and lysine-specific gingipain (Kgp) into OMVs. The treatment with *Pg* OMV attenuated the insulin signalling in HepG2 cells, and its effects were eliminated by OMVs from gingipain-deleated *Pg*. A Cy7 fluorescent signal was detected in the liver in mice injected with Cy7-labelled-*Pg* OMVs. The exposure of *Pg* OMVs for 3 weeks slightly increased casual blood glucose and insulin tolerance level in mice.

Summary/Conclusion: *Pg* OMVs packaging gingipains were delivered to the liver, resulting in the reduction of insulin sensitivity. These capabilities of *Pg* OMVs may contribute to the progress of diabetes mellitus.

PT09: Advances in EV Quantification and Characterization
Chairs: Randy Carney; Edwin van der Pol
Location: Level 3, Hall A

15:30–16:30

PT09.01

Extracellular vesicle concentrations in human plasma and serum as revealed by microfluidic resistive pulse sensing and size exclusion chromatography coupled with on-line fluorescence detection

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Introduction: Blood is one of the most important sources of EVs in biomarker applications. However, there is a huge variation in the reported values of EV concentrations in plasma and serum in the current literature. Therefore, there is a continuous demand for new techniques for accurate determination of EV concentration. The aim of this study was to characterize EVs in normal plasma and serum using novel techniques such as microfluidic resistive pulse sensing (MRPS) and size exclusion chromatography (SEC) coupled with on-line fluorescence detection.
Methods: To obtain cell free serum and plasma, blood was collected from healthy volunteers using serum activator and EDTA vacutainer tubes, respectively. Cells were removed by centrifugation at 2500 x g twice. Samples were further purified with a Sepharose CL-2B gravity column and analysed by MRPS using the nCS1 instrument (Spectradyne LLC, USA). For the fluorescence SEC experiments, samples were labelled with PE-conjugated anti-CD61 and analysed with a JASCO (Japan) liquid chromatography system supplemented with an FP-2020 fluorescence detector and using a 1 mL column filled with CL-2B gel.
Results: The particle concentrations of serum and plasma determined by MRPS in the 65–250 nm size range were 2.06E+10 1/mL and 1.77E+10 1/mL, respectively. In the 250–2000 nm range, we found 2.22E+8 1/mL and 5.50E+7 1/mL for serum and plasma. These concentrations correspond to 0.29 E+10 1/mL increase for the smaller size range, and 1.67E+8 1/mL for the larger size range, which can be accounted for the EVs produced during clotting. Fluorescence SEC experiments with PE-CD61 revealed that the percentage of CD61 bound to EVs increased from 2.25% (plasma) to 36% (serum). Using these data, we obtained that one platelet-derived EV contains approx. 15 CD61 glycoproteins in average.
Summary/Conclusion: By the combination of MRPS and fluorescence SEC we quantified the overall particle concentrations in serum and plasma, and using a platelet-specific fluorescently labelled antibody, we determined the average number of CD61 glycoproteins on platelet-derived EVs formed during blood clotting.
Funding: This work was supported under grant numbers PD 121326 and NVKP_16-1-2016-0007 by NKFIH (Hungary). ZV was supported by the János Bolyai Research Fellowship.

PT09.02

The nanobioanalytical platform, a tuneable tool for a sensitive detection & characterization of extracellular vesicles subsets from biological samples

Balasubramaniam Namasivayam[a], Yu-Wen Wu[b], Liling Delila[b], Annie Frelet-Barrand[a], Thierry Burnouf[b], Celine Elie-Caille[a] and Wilfrid Boireau[a]

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Introduction: The NanoBioAnalytical (NBA) platform is an established, calibrated and label-free system to characterize Extracellular Vesicles (EVs), without limitation in size, in different biological samples [1, 2]. NBA benefits were recently highlighted in latest MISEV guidelines [3]. The NBA platform combines biodetection and phenotyping of EVs subsets by immunocapture monitored by Surface Plasmon Resonance (SPR) on biochip, followed by EVs quantitation and sizing thanks to metrological evaluation by Atomic Force Microscopy (AFM). Our aim is to push the limit of the NBA to address clinical studies involving EVs.
Methods: We emphasise here the performance of the NBA platform for establishing its dynamic range and limit of detection (LOD) for blood derived EVs. Concentration of EVs was first determined in solution by Tunable Resistive Pulse Sensing; NBA sensitivity and reliability was then studied by SPR on biochips presenting a-CD41 antibody arrays. Finally, even on 1000-fold diluted samples, reliable and complementary information to SPR measurements on size distribution,

counting and shape deciphering could be obtained by AFM.

Results: Optimizing different factors (flow rate, density of receptors on the surface, etc.) enabled detection of blood derived EVs at dynamic range from 10^6 to 10^9 particles /mL on a-CD41 surface. The determination of the LOD of EVs and their subsets size distribution at different capture levels are currently in progress.

Summary/Conclusion: The NBA platform is modular and capable of detecting EVs reliably even in highly diluted samples. Such characterization and correlation studies are crucial for accurate and comprehensive characterization of EVs in biological samples with good reproducibility.

References

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3. Thery et al., JEV. 2018. 8;1535750

Funding: Region Franche-Comté 2017–2020.

PT09.04

Multi-parametric single vesicle analysis using an interferometric imaging platform

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Introduction: Current single vesicle analysis techniques like electron microscopy and atomic force microscopy require high expertise and are limited in throughput. Flow cytometry (FC), which is regularly used to for single cell analysis and sorting, has limited sensitivity in light scatter mode for detection of highly abundant populations of EVs smaller than a 100 nm. Recent publications show that the exosome average diameter is around 50 nm, which has been measured by super-resolution imaging, nanoFCM, and TEM. The more sensitive fluorescence-based detection of EVs is also difficult because EVs could have much less than 10 epitopes of the marker of interest, a limit for most FC systems.

Methods: To address the limitation in single vesicles analysis we have developed a technique that can size, enumerate, and co-localize 4 markers (surface and cargo) on single vesicles across 10 different subpopulations on a single sensor surface. The technique is termed SP-IRIS and commercialized as ExoView™ by NanoView Biosciences. EvoView™ relies on a bilayer substrate (silicon/silicon dioxide) that forms a common path interferometer for enhanced nanoparticle analysis. The calculated fluorescence detection limit approaches single fluorescence sensitivity established using fluorescent polystyrene nanoparticles (20–200nm diameter) corresponding to 180–110,000 MESF.

Results: A tetraspanin assay was developed on the ExoView™ platform for the detection of CD81, CD63, CD9 positive vesicles directly from cell culture samples without the need for purification. We can also permeabilize the vesicles to probe the cargo of individual vesicles. To validate the detection of tetraspanins and internal cargo proteins we used knockout cell lines as negative controls. The assay can also be used for detection of vesicles from other biological fluids like urine, plasma, CSF, and saliva. We demonstrated that most tetraspanin positive vesicles have a diameter around 50 nm, which agrees with TEM, versus the widely reported diameter of 100nm in the literature.

Summary/Conclusion: The ExoView platform is a scalable single vesicle analysis platform that can size, enumerate and run multi-parametric co-localization experiments directly from sample. The platform can be applied for basic research as well as biomarker discovery for liquid-biopsy applications.

PT09.05

Quantification of circulating extracellular vesicles from human plasma by utilizing a membrane-based microfluidic system

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Introduction: Extracellular vesicles (EVs) have served as biomarkers for cancer diagnosis and prognosis based on their carried cargos such as proteins and nucleic acids. To accurately and specifically quantify tumour-derived EVs from complex biofluids such as human plasma is potentially significant for precise diagnosis. Many techniques for EVs quantification have been developed in the past decade, including nanoparticles tracking analysis, total internal reflection fluorescence microscopy, flow cytometry and enzyme-linked immunosorbent assays (ELISA). However, bulky and expensive instruments are required for these approaches. Therefore, this study provides a simple and low-cost approach to quantify circulating EVs from human plasma by using the ELISA method and a fluorescent microscope on a membrane-based integrated microfluidic platform.

Methods: In this study, a membrane-based integrated microfluidic platform was used for EVs collection,

enrichment and fluorescent detection process. A track-etched membrane filter with a pore size of 0.03 μm that could enrich EVs and deplete small molecules during washing steps was packaged in a polydimethylsiloxane-based microfluidic platform. After EVs enriching, an on-chip ELISA assay was performed involving the following steps including (1) anti-CD63 antibody (EPR5702) incubation, (2) horseradish peroxidase (HRP) conjugated anti-rabbit antibody incubation, and (3) tetramethylrhodamine-labelled tyramide incubation. It is worth noting that tyramide molecules could be accumulated on the surface of EVs to amplify the fluorescent signal and observed under a fluorescent microscope. With this approach, absolute quantification of EVs with high specificity could be achieved.

Results: The experimental results showed that CD63-positive circulating EVs in human plasma could be individually observed under a fluorescent microscope. By using imaging software (ImageJ) to perform image analysis, the total number of EVs could be quantified such that the concentration of EVs in plasma could be measured.

Summary/Conclusion: The developed method could be used to quantify EVs with high specificity and could be widely used in most general laboratory for precise diagnosis of circulating EVs from human plasma.

Funding: Ministry of Science and Technology of Taiwan (MOST 106–2221-E-007–001, MOST 107–2221-E-007–013-MY3)

PT09.06

Electrophoretic separation of EVs using a microfluidic platform

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Introduction: Absence of adequate tools for analysing and/or identifying mesoscopic-sized particles ranging from tens to hundreds of nanometres is the potential obstacle in both fundamental and applied studies of extracellular vesicles (EVs), and hence, there is a growing demand for a novel analytical method of nanoparticles with good reproducibility and ease of use.

Methods: In the last several years, we reported the usefulness of electrophoretic mobility as an index for typing individual EVs based on their surface properties. To meet the requirement of separation and recovery of different types of EVs, we demonstrate the use of micro-free-flow electrophoresis (micro-FFE) devices for this purpose. Since the 1990s, micro-FFE devices have been developed to allow for smaller sample volume and reagent consumption. To solve several technical problems involving the generation of electrolysis gas on the electrodes, most of the micro-FFE devices reported in the past were fabricated using elaborate micromachining process on silicon or glass substrates. However, high-cost micromachining processes were required, and these were not suitable for mass production.

Results: Based on these backgrounds, we recently developed a polymer-based easy-to-fabricate micro-FFE device and overcame the problems mentioned above. In this presentation, we will introduce the application of this device to EV separations in this presentation. Electrophoretic separation of Sk-Br-3 derived exosomes expressed with HER2 antigen were demonstrated with and without the combination use of the anti-HER2 antibody for molecular specific separation.

Summary/Conclusion: The present method will be one of the promising candidates for separating favourable types of EVs from heterogeneous samples.

Funding: Center of Innovation Program (COI STREAM) from Japan Science and Technology Agency (JST)

PT09.07

Size distribution of extracellular vesicles by microfluidic resistive pulse sensing and small-angle neutron scattering

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Introduction: Accurate size determination of extracellular vesicles (EVs) is still challenging because of the detection limit and sensitivity of the methods used for their characterization. In this study, we used two novel techniques such as microfluidic resistive pulse sensing (MRPS) and small-angle neutron scattering (SANS) for the size determination of reference liposome samples and red blood cell derived EVs (REVs) and compared the obtained mean diameter values with those measured by dynamic light scattering (DLS).

Methods: Liposomes were prepared by extrusion using polycarbonate membranes with 50 and 100 nm pore sizes (SSL-50, SSL-100). REVs were isolated from red blood cell concentrate supernatant by centrifugation at 16.000 x g and further purified with a Sepharose CL-2B gravity column. MRPS experiments were performed with the nCS1 instrument (Spectradyne LLC, USA). SANS measurements were performed at the KWS-3 instrument operated by Jülich Centre for Neutron

Science at the FRMII (Garching, Germany). DLS measurements were performed using a W130i instrument (Avid Nano Ltd., UK).

Results: MRPS provided particle size distributions with mean diameter values of 69, 96 and 181 nm for SSL-50 and SSL-100 liposomes and for the REV sample, respectively. The values obtained by SANS (58, 73 and 132 nm, respectively) are smaller than the MRPS results, which can be explained by the fact that the hydrocarbon chain region of the lipid bilayer gives the highest scattering contribution in case of SANS, which corresponds to a smaller diameter than the overall size determined by MRPS. In contrast, DLS provided the largest diameter values, namely 109, 142 and 226 nm, respectively.

Summary/Conclusion: Size determination methods based on different physical principles can result in large variation of the reported mean diameter of liposomes and EVs. Optical methods are biased due to their size-dependent sensitivity. SANS can be used for mono disperse samples only. In case of resistive pulse sensing, the microfluidic design overcomes many practical problems accounted with this technique, and as a single particle, non-optical method, it is less affected by the above-mentioned drawbacks.

Funding: This work was supported under grant numbers PD 121,326 and NVKP_16-1–2016-0007 by NKFIH (Hungary). ZV was supported by the János Bolyai Research Fellowship.

PT09.09

Analysis of intracellular dynamics of exosomes and changes of surface markers

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Introduction: In the biological study, a standard method for observing natural interactions between cells is co-culturing technique. The existing co-culture research method is generally classified into two main groups depending on the state of adhesion between cells: direct co-culture or indirect co-culture. In indirect co-culture, standard methods for filter separation of cells include methods using vertical-insert type co-culture plate (VTCP) named after the structure or trademark (i.e. cell-culture insert, Transwell). These methods have been used in many studies thus far, its application to exosomes research has been limited. It is difficult to obtain high-quality images of cells in the upper culture chamber due to the short focal length of the microscope. We developed a novel cell culturing chamber (horizontal connection type co-culture plate; HTCP). HTCP made it possible to analyse intracellular kinetics and changes in surface markers of exosomes.

Methods: To examine the essential interactions of exosomes, we evaluated the uptake of extracellular exosomes using this HTCP. Culturing cells with GFP-labelled exosomes in only one container and detecting the presence of GFP in cells in the adjoining container.

Also, various chemicals were added, and analysis was made on changes in the kinetics of exosome and changes in surface markers.

Results: It was possible to confirm the exosome passed through the filter and to identify the origin of exosomes and to analyse the distribution of the exosome in the cells. We found that the amount of exosome secreted by cells increased by an agent. As a result of the analysis, although the amount of CD63 per one exosome was decreased, the amount of CD63 per one cell was increased.

Summary/Conclusion: This fact indicates that there may be no point in comparing the amount of protein or miRNA contained in exosomes. Detailed data will be presented at this workshop.

PT09.10

Protease biomarker detection using functionalized bioplastic-based biosensors

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Introduction: Extracellular vesicles (EVs) are potentially the “seeds”, that were famously metaphorized by Dr Stephen Paget in 1889 when he noted that particular primary tumours preferentially metastasized to particular organs. EV-associated metalloproteinases conceivably play important roles in priming metastatic sites. Indeed, many studies demonstrate the complex roles that metalloproteinases have in cancer biology. EVs can be readily accessed from patient liquid biopsies and an analysis of EV-associated metalloproteinase biomarkers may enable early-stage cancer detection.

Methods: In order to detect EV-associated metalloproteinases we developed a library of biosensors. These biosensors utilize PhaC-reporter fusion proteins that are bound to microbially manufactured bioplastic beads. These PhaC-fusions also incorporate specific metalloproteinase cleavage sites. In the presence of a specific metalloproteinase, the reporter protein is cleaved off the bioplastic bead – resulting in a loss of

bead fluorescence that can be measured using high-throughput flow cytometry. These biosensors were assayed using either recombinant proteinases or isolated EVs from *in vitro* cancer models.
Results: Human metalloproteinase recognition motifs were identified in the literature and a total of 70 different metalloproteinase biosensors were designed. A control biosensor (PhaC-112L-T-G) detected 0.5 U of tobacco etch virus protease (AcTEV) activity and the PhaC-112L-P14-G biosensor, despite some background off-target activity, was able to detect 0.033 mU of recombinant MMP14 activity. Membrane-bound metalloproteinases MMP14 and ADAM10 were also detected in EVs isolated (ultracentrifugation) from *in vitro* cancer models.
Summary/Conclusion: Our biosensors detected EV-associated metalloproteinases and could serve as useful research tools for EV-biomarker discovery.
Funding: Dr Richard Kelwick is funded by a Royal Society of Edinburgh Enterprise Fellowship and an Imperial Confidence in Concept 2018 grant. We also acknowledge the support of Engineering and Physical Science Research Council (EPSRC) grants [EP/L011573/1; EP/P028519/1] and the Biotechnology and Biological Sciences Research Council (BBSRC) Foundry grant [BB/L027852/1].

PT09.11

Single exosome size analysis using super resolution microscopy

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Introduction: Exosomes are a type of extracellular vesicle (EV) with diameters of 30–150 nm and are secreted from most cell types. Owing to their significant role as cellular messengers and potential applications in disease detection, treatment and targeted delivery, growing efforts have been made in this relatively new field. However, exosome research is hindered by significant challenges including inefficient separation methods, difficulties in characterization and lack of definitive biomarkers. Particularly, exosomes are difficult to visualize since their small size falls below the resolution limit of conventional microscopes (~200 nm).
Methods: Recent progress in super-resolution has provided novel tools in exosome characterization. In this study, we present a single platform to capture pre-coarsely isolated exosomes onto an imaging flow chamber through specific anti-bodies and perform super-resolution imaging on the same device. Specifically, the surface of the imaging chamber is passivated with anti-CD 63 to capture the DiD stained vesicles. The acquisition of the raw image series was done using total internal reflection fluorescence microscopy (TIRF) with a 642-nm diode laser for excitation. Two types of super-resolution techniques were tested including super resolution radial fluctuations (SRRF) and stochastic optical reconstruction microscopy (STORM).
Results: The size of single exosomes in the final images were estimated by the full-width at half-maximum (FWHM) of Gaussian fitted to the distribution of single molecules. We have found that the resolution limit of the single particle is reduced to 70 nm. The preliminary data from SRRF and STORM showed the particle size and size distribution were compared to nanoparticle tracking analysis (NTA) results.
Summary/Conclusion: This method provides in-depth size analysis of single exosomes below the diffraction limit. Additionally, capturing exosomes from coarsely isolated samples via specific antibodies would reduce the time required for sequential ultracentrifugation, the current standard technique for exosome isolation. Finally, this imaging chamber presents a versatile platform for protein profiling as the captured exosomes can be labelled with specific antibody-dye conjugates to reveal the surface proteins contents.

PT09.12=OWP3.09

Identification of single tumour-derived extracellular vesicles by means of optical tweezers and Raman spectroscopy

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Introduction: EVs derived from cancer cells play a role in tumour cell proliferation, migration, invasion and metastasis. Their presence in body fluids, such as blood, makes them potential biomarkers for cancer disease. However, the identification of single tdEVs can be challenging due to their heterogeneity, their ultra-small size, their size overlap with many other normal EVs and contaminants in body fluids and the lack of knowledge on their chemical composition.
Methods: Synchronized optical tweezers and Raman spectroscopy have enabled a study of individual EVs. The new method detects individual trapping events from Rayleigh scattering. The synchronous recording of Raman scattering enabled the acquisition of Raman spectra of both individual and multiple EVs, disclosing

their chemical composition. Furthermore, Mie light scattering theory has been used to relate the Rayleigh scattering intensity to the size of trapped EVs.

Results: The light scattered of trapped EVs gave rise to step-wise time traces that can be used to distinguish individual trapping events from accumulative cluster events due to the discrete nature of the steps which correspond to single trapping events. Next, we confirmed the trapping of individual EVs derived from PC3 cells, red blood cells, platelets and blood plasma by acquiring both, Rayleigh and Raman scattering signals. While the step-wise trend in the Rayleigh scattering signal suggests trapping of single particles, the Raman scattering signal demonstrates the nature of the trapped EVs. Through principal component analysis (PCA), the main spectral variations among the four EV types were identified. The principal component scores grouped the PC3-derived EVs in a separate cluster from the rest of the EVs.

Summary/conclusion: We have developed an automated single particle optical tweezers – Raman and Rayleigh scattering setup to trap and release single EVs over time. We demonstrated single-EV trapping by simultaneous acquisition of Rayleigh and Raman scattering. PCA enabled the identification of single-EVs derived from the cancer cell line PC3. This discloses chemical information as a step towards the identification and characterization of single tumour-derived EVs in blood.

Funding: Cancer ID – project number 14193, (partially) financed by the Netherlands Organisation for Scientific Research (NWO)

PT09.13=OWP3.02

Immunocapturing of tumour-derived extracellular vesicles on micropatterned and antibody-conjugated surfaces for individual correlative light, probe and electron measurements

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Introduction: Tumour-derived extracellular vesicles (tdEVs) are promising biomarkers for cancer patient management. The screening of blood samples for tdEVs shows prognostic power comparable to screening of tumour cells. However, due to the overlap in size between tdEVs, non-cancer EVs, lipoproteins and cell debris, new approaches, not only based on size, are required for the reliable isolation of tdEVs and their quantification. We report an integrated analysis methodology to study single tdEVs using correlative data from scanning electron microscopy (SEM), Raman imaging (RI) and atomic force microscopy (AFM) to obtain a comprehensive dataset allowing identifying features unique to tdEVs.

Methods: Indium tin oxide (ITO)-coated fused silica was selected for its low Raman background. Substrates (1 x 1 cm^2) featuring position-dependent markings ("navigation marks") patterned by photolithography were modified with a monolayer of amino dodecyl phosphonic acid. The amine moieties were next reacted with poly(ethylene glycol) diglycidyl ether, forming an anti-biofouling layer. Anti-EpCAM antibodies were subsequently covalently bound on this surface. Samples of both tdEVs obtained from LNCaP cell lines and RBC-derived EVs were then introduced to the surfaces. Finally, non-specifically bound EVs were washed away before SEM, AFM and Raman measurements were performed.

Results: Multiple objects were captured on the fully functionalized ITO surfaces, according to SEM imaging, while in negative control experiments (lacking functionalization or lacking antibody or using EpCAM-negative EVs), no object was detected. Principal component analysis of their Raman spectra, previously demonstrated to be able to distinguish tdEVs from RBC-derived EVs, revealed the presence of characteristic lipid bands (e.g. 2851 cm^{-1}) in the captured tdEVs. AFM showed a surface coverage of ~4 × 10^5 EVs per mm^2 with a size distribution similar to that found by NTA.

Summary/conclusion: A platform was developed for multi-modal analysis of selectively isolated tdEVs for their multi-modal analysis. In the future, the scope of this platform will be extended to other combinations of probe, light and electron microscopy techniques to relate additional parameters describing the captured EVs.

Funding: Funded by NWO Perspectief

PT09.14=OWP3.03

The development of a scalable extracellular vesicle subset characterization pipeline.

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Introduction: Liquid biopsies offer an important alternative to tumour biopsies that may be limited by the challenges of invasive procedures. We hypothesize that

circulating Extracellular Vesicles (EVs) and their cargo may provide a useful surrogate biopsy method. Due to their small diameter (30-1000 nm), EVs migrate from tissue into the peripheral circulation and provide a snapshot of the producing cells. Our lab has developed a first-in-class pipeline to use single cell – omics methods to characterize EV heterogeneity with high-sensitivity by combining multiplex assays and our custom MultiPlex Analysis post-acquisition analysis software (MPA_{PASS}), with subsequent high-resolution, single EV flow cytometric (FCM) methods.

Methods: A standalone software package was developed in MATLAB to allow importation of multiplex flow cytometry output data. The package enables data quality screening of detection antibodies, bead recovery and data normalization methods. The software is equipped to handle large data sets comprising hundreds/thousands of phenotypes and samples. Data can be visualized in a variety of ways along with clustering using multidimensional data analysis techniques. All software outputs can be exported in a standardized templates containing metadata for reporting, as well as uploaded into atlases such as Genboree, where multiplex data can be stratified by RNAseq datasets. Analysis using this pipeline has been conducted using human samples from a variety of mediums including CSF, serum, and plasma comparing EV phenotypes.

Results: Our multiplex approach and MPAPASS software allows the use of single cell -omics tools for EV subset analysis in manner that will elucidate the biological significance and function of different types of EVs. This high-throughput pipeline evaluates hundreds of EV protein profiles and will allow evaluation of millions of RNA:protein profiles in an unprecedented manner. Integration of RNA sequencing with protein characterization could provide an entirely new way of understanding EV regulation and function.

Summary/conclusion: Our data show this form of EV profiling provides a way to monitor clinical responses early in the course of treatment, which may ultimately improve patient care and outcomes.

PT10: EVs and Stem Cells
Chairs: Takashi Asada; Myung-Shin Lee
Location: Level 3, Hall A

15:30–16:30

PT10.01

3D culture of dental pulp pluripotent-like stem cells (DPPSC) improves their pluripotency and provides a serum-free culture condition for exosome production

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Introduction: Exosomes from stem cells have been identified as a novel cell-free therapeutic for regenerative medicine. Culturing them in a serum-free condition for exosome isolation still poses a major challenge. This work focused on the establishment of a 3D culture of Dental Pulp Pluripotent-like Stem Cells (DPPSC) – a newly characterized pluripotent-like stem cell from adult tissue, for exosome production.
Methods: DPPSC were initially cultured in monolayer (2D) in their basal medium with four different supplementations: human serum (HS), exosome-depleted human serum (ED-HS), and two different serum replacements (SR1 & SR2). Morphology and growth rate of cells were analysed by bright-field microscopy and regular cell counting. DPPSC were then transferred to a microwell culture plate for 3D culture in the four differentially supplemented media and maintained for 24 days. Spheroid formation and morphology was observed throughout culture using bright-field microscopy. Spheroids were harvested on Day 24 and the expression of pluripotency genes Oct4A and Nanog were analysed by qPCR. Vesicles isolated from DPPSC conditioned-medium were characterized for size, yield and exosomal markers using Nanoparticle Tracking Analysis (NTA) and dot blot.
Results: In 2D culture, only DPPSC cultured in the default HS medium proliferated and showed the expected morphology. In 3D culture, DPPSC in SR1 medium formed spheroids of similar morphology and size to that of HS medium. Significantly smaller spheroids were formed by DPPSC in ED-HS medium, while DPPSC barely formed spheroids in SR2 medium. qPCR analysis showed that while expression of Oct4A gene in DPPSC cells from 2D and 3D culture (both in HS and SR1 media) was similar, expression of Nanog in DPPSC spheroids in SR1 medium was significantly higher than the spheroids in HS medium and the cells from 2D culture. Vesicles isolated from DPPSC spheroid in SR1 conditioned medium from Day 1–12 and Day 13–24 of culture showed sizes that fall within the exosomal size range, and are positive for the exosomal markers CD81, CD9 and CD63. Vesicle yield for Day 13–24 was higher than that of Day 1–12, but a larger percentage of particles from the latter were positive for the three exosomal markers.
Summary/Conclusion: 3D spheroid culture of DPPSC in SR1 medium showed improvement in pluripotency, and allows for a serum-free culture for exosome production.

PT10.02

Increased exosome secretion is essential for myeloma stem cells to survive in hypoxic condition

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Introduction: Cancer stem cells (CSCs) of the highly tumorigenic cell population are critically associated with the poor prognosis of patients in various types of cancer. In our previous study, the multiple myeloma (MM) cells which were chronically cultured in a hypoxic condition (over 6 months, 1% oxygen) exhibited stem cell characteristics. It suggests that MM stem cells are capable of adapting to hypoxic stress although the adaptation mechanism remains unclear. We focused on the excessive secretion of exosomes from hypoxia-adapted MM cells (HA-MM cells). Exosomes are considered as a garbage bin to remove unnecessary molecules from the cytoplasm to maintain cellular homeostasis, as well as a novel intercellular communication tool.
Methods: GW4869, an inhibitor of the ceramide-mediated inward budding of the multivesicular bodies for exosome biogenesis, was applied to analyse the response to a deficiency of exosome secretion from their reduced production in HA-MM cells.
Results: GW4869 increased the rate of Annexin V positive (apoptotic) cells and induced the expression of fragmented PARP in HA-MM cells, but not in

parental cells cultured in a normoxic condition (20% oxygen). With the addition of HA-MM-derived exosomes, GW4869-induced apoptosis was not attenuated. From these results, HA-MM cells are likely to release exosomes to maintain the intracellular environment in a state of homeostasis, but not to receive them for autocrine signal. Hexokinase 2 (HK2) generates glucose-6-phosphate, which is further metabolized by both the glycolytic pathway and the pentose phosphate pathway (PPP). PPP plays a major role in supplying NADPH for detoxification of intracellular reactive oxygen species (ROS). The upregulated HK2 protein expression in HA-MM cells was diminished by GW4869. With dichlorodihydrofluorescein staining assay, GW4869 increased intracellular ROS production in HA-MM cells. Thus, the failure of exosome secretion might alter the energy metabolism leading to ROS-associated apoptosis.

Summary/Conclusion: Enhancement of exosome secretion is a survival strategy for hypoxic adaptation in MM stem cells. This study could provide a critical insight to develop a novel strategy for CSC-targeted therapy.

Funding: MEXT-Supported Program for the Strategic Research Foundation at Private Universities

PT10.04

Extracellular vesicles released from human iPSC-derived 3D retinas contain small RNAs with roles in development and differentiation

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Introduction: Noncoding small RNAs in the retina regulate gene expression by targeting and repressing mRNA. Small RNAs are secreted in extracellular vesicles (EVs). Analysis of EVs released from developing retinal tissue is an essential step in elucidating the role of EV molecular cargo and signalling during retinogenesis. A number of canonical genes are associated with retinal cell fate determination during development, but EV-mediated gene regulation in the retinal microenvironment remains undefined. In this study, we characterize the microRNA, tRNA, and piRNA composition of EVs secreted from human induced pluripotent stem cell (hiPSCs) – derived 3D retinas at three developmental time points that correlate with hallmarks of retinal cell differentiation and lamination *in vivo*.

Methods: Retinal organoids were generated from hiPSCs. We selected three developmental time points (day 42, 63 and 90) that represent distinctive stages during normal retinal cell fate specification and lamination. We analysed the release rate, concentration, morphology and content (miRNA, tRNA and piRNA) of EVs released from human hiPSCs-derived 3D retinas.

Results: The genetic signalling, developmental time course and morphogenesis of these retinal organoids were comparable to those of developing human retinas *in vivo*. According to Gene Ontology analysis, miRNA targets at the earliest stage of development were more relevant to early differentiation and cell morphogenesis, whereas miRNA targets at the later stages were more relevant to cell proliferation, cell differentiation, and cell migration.

Summary/Conclusion: For the first time, this work demonstrates the rate of release and concentration of EVs from developing hiPSC-derived 3D retinal tissue. We report a large variety of small RNAs in EVs from hiPSC-derived 3D retinas, including miRNAs, tRNAs and piRNAs. The full range of small RNAs detected in our EVs may act as regulatory elements to modulate gene activity and may serve as biomarkers of normal development. This work represents the first sequencing analysis of small RNA species contained in hiPSC-derived 3D retinas and their released EVs.

Funding: NIH (R21 EY026752, SC3 GM113782); Research to Prevent Blindness; Challenge Grant to the Department of Ophthalmology, CU; NIH/NEI R01EY022631.

PT10.05

Xeno-free manufacturing of MSC-EVs in scalable bioreactor culture

Katrina Adlerz[a], Josephine Lembong[a], Tim Olsen[a], Jon Rowley[a] and Taby Ahsan[b]

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Introduction: There have been over 800 clinical trials using mesenchymal stem/stromal cells (MSCs) for therapeutic applications. Due to their similar therapeutic effects to MSCs and potential as a key bioactive agent in regenerative medicine applications, MSC-

derived extracellular vesicles (MSC-EVs) are being increasingly investigated as a clinical therapy for a broad range of indications. It was recently found that the number of exosomes released from 2M MSCs in 48 h is equivalent to a single dose for a rodent. Hence, most indications would require a MSC production lot size that is hardly achievable in 2D culture. Therefore, larger scalable bioreactor systems will be crucial to generate enough EVs for clinical doses. This study developed a protocol for xeno-free (XF) scalable MSC-EV manufacturing and compared MSC-EV characteristics from 2D culture and various bioreactor scales.

Methods: Human Bone Marrow-Derived MSCs (hBM-MSC) were cultured on microcarriers in suspension using 0.1, 3 and 15L bioreactors. After cell inoculation, a bioreactor feed was added on Day 3, and cultures switched to an EV collection media on Day 4. After 1, 2 and 3 days, the collection medium was analysed for metabolites, particle size, and particle concentration. The MSC-EVs in the conditioned medium was also evaluated for protein expression of Alix, CD63, and CD81, RNA expression and wound healing capability.

Results: A protocol was developed for manufacturing MSC-EVs in an XF fed-batch bioreactor culture, resulting in cell yield of >0.5M cells/mL within 4 days. We demonstrated that this process was directly scalable from the small (0.1L) to development (3L) and pilot scale (15L) bioreactors, maintaining similar cell density. To remove the residual particles from the expansion media, an additional wash step before the switch to the collection media was required in bioreactors. With a similar collection process and comparable cell density, we expect to see consistent EV production per cell at these different scales of manufacturing systems.

Summary/Conclusion: Optimizing EV yield will become increasingly important as EVs become used in the clinic. We have developed a scalable fed-batch process for large scale expansion of hMSCs and a protocol for EV production in suspension bioreactors.

Funding: This work was funded by RoosterBio, Inc.

PT10.06

Proteomic analysis of extracellular vesicles from MSC cultured with stroke serum

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Introduction: Serum from stroke patients increases mesenchymal stem cells trophism towards the infarcted brain area and increased the proliferation rate and the neurorestorative capacity of MSCs. Our previous study confirmed that intravenous infusion of MSC-EVs is more advantageous than MSCs as a safe treatment. EVs are lipid enclosed vesicular structures that contain bioactive RNAs, DNA and proteins, which possess therapeutic molecules similar to MSCs. Therefore, we hypothesized that the certain proteins containing EV released from stroke serum cultured MSCs may affect the neurogenesis and angiogenesis of recipient cells.

Methods: EVs were purified from conditioned media of MSC cultured with FBS (FBS-MSC) and MSC cultured with stroke serum (SS-MSC). These EVs were characterized by nanoparticle tracking analysis. EV protein profiling in conditioned media was systematically compared through utilizing LC-MS/MS-based label-free quantification. Real-time PCR was performed to determine the difference in the gene expression in each cell. The protein concentration in the EV was confirmed by ELISA.

Results: A total of 1068 proteins were identified from SS-MSC-EV and FBS-MSC-EV through LC-MS. According to statistical analysis, 22 proteins were found to be more than 2-fold ($p < 0.05$) upregulated in SS-MSC_EV. ITGA5, CLU, and CTSB were significantly increased of SS-MSC gene expression levels compared to FBS-MSC. Among the candidate proteins, clusterin (CLU) was found to be upregulated in EVs from SS-MSC compared to those from FBS-MSC.

Summary/Conclusion: These results suggest that SS-MSC_EVs containing clusterin may promote intercellular communication and affect neurogenesis and angiogenesis of recipient cells.

Funding: This study was supported by a grant from the Korean Healthcare Technology R&D Project, Ministry of Health & Welfare (HI17C1256) and Basic Science Research Program, the Ministry of Science, ICT and Future Planning (2018M3A9H1023675).

PT10.07

Adipose-derived Stem/Stromal Cell secretome, containing both soluble factors and extracellular vesicles, exerts chondroprotective effects *in vitro*

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Introduction: Up to now several clinical trials have shown the safety and efficacy of the intra-articular injection of Adipose-derived Mesenchymal Stem/ Stromal Cells (ASCs) in contrasting osteoarthritis.

Since ASCs act predominantly through paracrine mechanisms, their secretome represents a promising cell-free alternative. Here we identified anti-hypertrophic and anti-catabolic effects of ASC conditioned medium (ASC-CM) on TNFα-stimulated human primary articular chondrocytes (CHs).
Methods: CHs were treated with 10 ng/mL TNFα and/or ASC-CM administered at a 1:5 recipient:donor cell ratio. Cell viability was assessed up to day 9. The activity, expression and/or release of hypertrophy markers (MMP-13, Collagen X and Osteocalcin), catabolic mediators (MMP-3) and cartilage-protective factors were assessed up to day 3 by enzymatic assays, qRT-PCR, Western Blot and multiplex immunoassays.
Results: ASC-CM blunted TNFα-induced hypertrophy, reducing the enhanced levels of MMP-13 activity (−61%), Osteocalcin (−37%) and Collagen X (−18%). In addition, also MMP-3 activity was diminished by −59%. We associated the observed reduction of MMP-3 and MMP-13 activity to the abundancy of TIMPs (Tissue Inhibitors of MMPs) in ASC secretome, rather than to a direct down-modulation of their expression and/or release. Moreover, ASC-CM contains high levels of OPG and DKK-1, other known chondroprotective factors.
Summary/Conclusion: ASC-CM is rich in cartilage-protective factors and exerts anti-hypertrophic and anti-catabolic effects on TNFα-stimulated CHs. These evidences open the way for its possible clinical use as a cell-free approach in contrasting osteoarthritis. We are currently investigating through a differential proteomic analysis if the recognized chondroprotective effectors are enriched in the vesicular rather than the soluble component of the secretome.

PT10.08

Epigenetic alterations in mesenchymal stem cells by osteosarcoma derived extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are central to intercellular communication and play an important role in cancer progression and development. Osteosarcoma (OS) is an aggressive bone tumour, characterized by presence of malignant mesenchymal cells. Specific tumour-driving genetic alterations associated with OS development are poorly understood. The cell of origin for OS also remain unknown but cells of the mesenchymal stem cell (MSC) osteogenic lineage are likely candidates, thus indicating that MSCs and the OS stroma cells may be related cell types. Therefore, this study set out to examine the contribution of EV-mediated intercellular crosstalk of MSC and OS.
Methods: MSCs and pre-osteoblasts were treated with OS-EVs at different time points, and the epigenetic signature of OS-EVs was assessed by LINE-1 and tumour suppressor genes methylation analysis. In addition, surface markers and gene and expression of specific genes related to bone microenvironment remodelling (MMP1, VEGF-A, ICAM1) were also evaluated.
Results: Our data indicated that OS-EVs mediated LINE-1 hypomethylation in MSCs, whereas an opposite effect was seen in pre-osteoblast, indicating that MSCs but not pre-osteoblasts were more susceptible to epigenetic transformation. Thus, OS-EVs dictated the fate of MSCs by modulating the epigenetic status, and also influenced the expression of genes related to bone microenvironment remodelling.
Summary/Conclusion: Overall, this study provided evidence that epigenetic regulation may appear to be an early event in the transformation of MSCs. Elucidating the mechanisms of EV-mediated communication may lead to new avenues for therapeutic exploitation.
Funding: This research was supported by University of Helsinki project funding (WBS490302, WBS73714112), Helsinki University Hospital State funding for university-level health research (Y1014SUL05, TYH2016130), the Finnish Dental Society Apollonia, Egyptian Ministry of Higher Education (MoHE) and Selma and Maja-Lisa Selander foundation.

PT10.09

Comprehensive proteomics and microRNA analyses of adult neural stem cell derived exosomes after stroke

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Introduction: Neural stem cells (NSC) are known to facilitate healing of ischemic brain tissues. Recent studies show that NSC derived exosomes function as paracrine effectors to promote neurovascular remodelling including angiogenesis and axonal outgrowth after stroke; nevertheless, the contents of the non-stroke and post stroke NSC exosome proteome and miRNA cargo have not been determined.

Methods: NSC derived exosomes were purified from conditioned media of cultured NSCs harvested from the subventricular zone of non-ischemic and ischemic rats, respectively. Liquid chromatography mass spectrometry (LCMS) and miRNA array were employed to comprehensively characterize the protein and miRNA contents of NSCs and their derived exosomes after stroke. Bioinformatic analyses were performed using Ingenuity Pathway Analysis (IPA).
Results: Exosome markers including CD63, CD9, Alix and size distribution (50–200nm) were verified with Western blot, transmission electron microscopy (TEM) and Nanosight, respectively. In total, proteomics analysis yielded 2409 and 1770 proteins identified in ischemic NSC and NSC derived exosomes, respectively. Bioinformatics analysis identified that 52, 39 and 31 proteins in the NSCs-derived exosomes were related to regulating neuronal cell proliferation, migration and differentiation, respectively. In addition, 318 miRNAs were identified in ischemic NSCs with 26% of miRNAs (84 miRNAs) overlapped with parent NSCs. Gene ontology analysis showed that up- and down-regulated miRNAs with the fold change above 1.5 were highly related to inflammation, invasion, cell proliferation, cell cycle, cell death, differentiation, etc. The top three upregulated miRNAs were validated in ischemic NSC-exosomes using real-time RT-PCR.
Summary/Conclusion: Collectively, the results of our proteomic and miRNA analysis, to our knowledge, demonstrate for the first time that NSC derived exosomes contain a robust profile of protein and miRNA effectors. These data provide a platform for beginning to understand the mechanism by which NSCs are activated after cerebral ischemia, and may lead to a deeper mechanistic understanding of their role in tissue repair after neural injury.
Funding: NIH RO1 DK102861, American Heart Association (AHA) grant 18IPA34170331, NINDS RO1 NS075156 and RO1 NS088656.

PT10.10

Anion exchange chromatographic isolation of iPSC-MSC derived extracellular vesicles ameliorated allergic asthma in mice

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Introduction: The extracellular vesicles (EVs) derived from mesenchymal stem cells have been shown to elicited similar therapeutic effects to their patent cells in many diseases. However, the difficulties in the large-scale preparation of high-purity EVs largely limited its clinical application in the future. We sought to apply a novel anion chromatography for the isolation of iPSC-MSC EVs, and explored the effects and mechanisms of iPSC-MSC EVs in the therapy for asthma.
Methods: The EV-enriched supernatants were collected for the isolation of the iPSC-MSC EVs using the anion chromatography. The morphologies of EVs were characterized by transmission electron microscope, the markers of EVs were assayed by western blot and flow cytometry. The anti-inflammatory effects of the EVs were determined using the macrophage assay. Also, the uptake activities of macrophages on RPF-iPSC-MSC EVs were determined. Finally, the asthma mouse model was developed and the iPSC-MSC EVs were administrated intravenously. The lung pathology, the levels of inflammatory cytokines in the bronchoalveolar lavage fluids (BALF) and the polarization of lung macrophages were evaluated.
Results: We successfully obtained concentrated iPSC-MSC EVs after the isolation and the final concentration of EVs was about 200 μg/mL (Bradford) and 10–$15*10^{11}$/mL (Nanosight). The iPSC-MSC EVs were morphologically intact and were positive for the markers including CD9/63/81, Alix and TSG101. Most of the preparations of iPSC-MSC EVs could significantly decreased the level of IL-6 in the macrophage assay. The Raw 264.7 macrophages began to uptake iPSC-MSC EVs at 4 h and the uptake activities peaked at 12 h and then receded at 24 h. Also, our *in vivo* study showed that splenic macrophages started to uptake iPSC-MSC EVs at 4 h and the uptake activities were augmented at 24 h. In addition, the iPSC-MSC EVs significantly reduced the inflammatory infiltration, the epithelial goblet cell numbers, the levels of inflammatory cytokines and inflammatory cells in the BALF as well as the polarizations of pulmonary macrophages.
Summary/Conclusion: Our results showed that the anion exchange chromatography was a promising method for the large preparation of iPSC-MSC EVs, which could possibly be an alternative therapy for asthma in the future.
Funding: NSFC (81322012, 81373174, 81471832, 81671882 and 81770984)

PT10.11

Characterization and miRNA expression profiles of exosomes from HLA homozygous haplotype dental pulp cells and iPS cells

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Introduction: Human leucocyte antigen (HLA) has played an important role to distinguish between self and non-self in the immune system. HLA homozygous cell of the multi-locus has been considered to be less likely to be rejected in the allograft. In recent years, the diameter to 100 nm of the extracellular vesicles called exosomes, to cellular functions secreted from iPS cells or tissue stem cells, partially responsible, such an immune response and during communication tools it has been reported to play a role as a tissue repair. In this study, we compared the exosomes from HHH-DP HLA homozygous haplotypes from cell-derived HHH-iPS cells (HHH) pulp (DP) cells and exosomes.

Methods: Three lines of HHH-DP cells established at Gifu University and HHH-iPS cells derived from these cells were used. DP and iPS cells were cultured in serum-free conditions. Exosomes were purified from culture supernatants by ultracentrifugation. Purified exosomes were subjected to particle size determination with a nanoparticle analysis system (Nanosight LM10), exosome markers and HLA class I evaluation by Western blotting (WB), and miRNA expression analysis, and results were compared. HHH-iPS cell exosomes were also examined if teratomas were formed in immunodeficient mice.

Results: Nanosight LM10 confirmed that the particle size peaks were nearly identical at ~100 nm. WB revealed that both DP cell exosomes and iPS cell exosomes expressed CD81 and HLA class I, but expression levels of CD81 and HLA class I were lower in iPS cell exosomes. The miRNA analysis showed that some miRNAs differed between cells and between exosomes. In assessment of teratoma forming ability, no tumour formation was observed.

Summary/Conclusion: HHH-DP cell exosomes and HHH-iPS cell exosomes were found to have different surface antigens and miRNA expression profiles. HHH-iPS cell exosomes showed a reduced level of HLA expression and no teratoma formation, and thus are potentially useful for therapeutic purpose.

PT11: EV Based Cancer Therapeutics
Chairs: AC Matin; Eva Rhode
Location: Level 3, Hall A

15:30–16:30

PT11.01

Cellular and secreted extracellular vesicles-encapsulated miRNAs in the 4T1 murine model of breast cancer

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Introduction: Extracellular vesicles (EVs) are secreted by all cells and are known to contain a range of genetic material such as microRNAs (miRNAs). EVs have been implicated in mediating intercellular communication to support breast cancer progression and also highlighted as a potential biomarker of disease. This study aimed to investigate the miRNA profile of EVs released by 4T1 breast cancer cells *in vitro* and to relate this to the circulating EV profile of an animal model of this disease.
Methods: 4T1 cells were cultured in EV-depleted media, and secreted EVs isolated through sequential differential centrifugation, micro-filtration and ultracentrifugation. EVs were also isolated from the sera of balb/c mice bearing 4T1 tumours. EVs were characterized by Nanoparticle Tracking Analysis (NTA), Western Blot and Transmission Electron Microscopy (TEM). RNA was extracted from all cells and EVs using the MagNA pure compact and Next-Generation Sequencing (NGS) targeting miRNA was performed. Targets of interest were validated by Polymerase Chain Reaction (PCR).
Results: EVs were successfully isolated from all samples with the majority of vesicles falling within the range of exosomes (30–120 nm). Western blot analysis confirmed the presence of tetraspanins CD63, CD81 and CD9. The characteristic size and shape (cup) of EVs were visualized by TEM. Over 380 previously annotated miRNAs were detected in the 4T1 secreted EVs, with 11 novel putative miRNA sequences identified. Twenty-five miRNAs were found to be differentially expressed between the cells and their secreted EVs. Interestingly, of these, 14 miRNAs were present at a significantly higher level in the EVs compared to the cells. Including a range of miRNA previously associated with cancer progression, e.g. miR-486-5p. Gene ontology enrichment identified a range of key biological processes that could potentially be regulated by the EV-miR profile detected such as tumour proliferation and bone cell resorption.
Summary/Conclusion: Analysis of EVs from animals bearing 4T1 tumours is ongoing to determine whether the EV-miR profile could serve as a biomarker of disease. The data presented demonstrates the selective packaging of tumour associated miRNAs into EVs which could play an important role in disease progression.
Funding: Irish Research Council, Government of Ireland Postgraduate Scholar 2016 GOIPG/2016/978.

PT11.03

Delivery of miR-185 enriched EVs from MSCs inhibits the progression of OPMD

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Introduction: Oral leucoplakia is one of the most common oral potentially malignant disorders (OPMD) and its malignant transformation is associated with chronic inflammation. It is clear that the tumour microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the fostering proliferation, survival and migration. Extracellular vesicles (EVs) shuttle complex molecular cargo between producer and recipient cells resulting in epigenetic regulation of cell function. EVs derived from mesenchymal stem cells (MSCs) have been found to promote therapeutic activities that are comparable to MSCs themselves.
Methods: Bone marrow derived MSCs were transfected with high copy numbers of miR-185 mimics and EVs were harvested using Genexosome Isolation kit. miR185 enriched EVs were characterized and applied on the buccal mucosa in the OPMD model exposed to 7,12-dimethylbenz anthracene (DMBA). Pathological analysis of the buccal mucosa was studied, and the topical and serum levels of inflammatory cytokines

and chemokines were measured. In addition, the expression levels of caspase 3 and 9 were examined.

Results: EVs released from genetically modified MSCs had ~25-fold higher expression levels of miR-185 than the control. Confocal microscopic imaging revealed that the PKH26 fluorescence labelled EVs principally localized in the buccal mucosa after administration. After treatment with miR-185 enriched EVs for 3 or 5 weeks, the topical inflammation severity in buccal mucosa was remarkably attenuated, the levels of IL-6, IL-1β, JE, MIP-1_a, MIP-2 and TREM-1 were decreased, and the numbers of inflammatory cells were reduced as well. Pathological analysis of the buccal tissue showed significantly decreased numbers of cells with hyperplasia or dysplasia after treatment. In addition, miR185 enriched EVs led to significantly increased levels of caspase 3 and 9 in the buccal tissue, indicating miR185 promotes the activation of apoptotic pathway.

Summary/Conclusion: miR-185 enriched EVs from MSCs are anti-inflammatory and anti-proliferative, and promote apoptosis. Genetically modified MSC-derived EVs have significant potential as a novel therapy for oral leucoplakia.

PT11.04

Identification of exosome secretion inhibitor for cancer therapy

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Introduction: Exosomes are nanosize secreting vesicles that can internalize and interact with other cells to initiate physiological and pathological signalling pathways. Especially, tumour-cell derived exosomes (TDXs) activate tumour-related mechanism such as proliferation, metastasis and drug resistance. We hypothesized that inhibition of exosome secretion may have beneficial effects in the treatment of cancer. Here, we found an old drug which inhibits exosome secretion from various cancer cells.

Methods: Human breast cancer and Human melanoma cancer cell lines were cultured. Immunoblotting was performed with primary antibodies against RAB27A and beta-actin. Cells were seeding in 24 well plates then treated candidate drugs for 24 h. Cell viability was measured by MTT assay. Exosomes were isolated by serial centrifugation method, then resuspended in PBS for further experiments. Exosome concentration was analysed by NTA.

Results: Exosome secretion was significantly decreased by drug treatment. In addition, this drug affected protein expression of RAB27A in various cancer cell lines. Moreover, migration and invasion activity of cancer cells were markedly suppressed by drug, suggesting that this drug has possibility to be used for anticancer therapy.

Summary/Conclusion: These findings demonstrate that a drug to inhibit exosome secretion selectively in cancer cells could be used for the therapy of various cancers. Importantly, our study offers a new mechanistic insight into drug development by the inhibition of exosome secretion.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (2014R1A5A2009242)

This research was supported by the Bio &Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of science & ICT (2017M3A9G8083382)

PT11.05

Platelet-derived microparticles as an oriented bullet for cancer treatment

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Introduction: Platelets (PLTs) and PLTs-derived microparticles (PMPs), released by PLTs upon thrombin activation, interact closely with cancer cells in the tumour microenvironment. Some researchers have been used synthetic nanoparticles loaded with anticancer agents and coated with whole PLT membranes for cancer therapy. However, isolating PLT membranes and synthesizing nanoparticles coatings sufficient for translational applications. Further, procedures for isolating PLT membranes may denature proteins, which may alter targeting specificity and incur an adverse risk of immunogenicity in patients. Therefore, our aim is to isolate and evaluate the ability of PMPs to serve as Trojan Horse carriers of anticancer drugs for cancer treatment.

Methods: PLT concentrates were centrifuged at 3000 × g for 15 min at 24 ± 3°C and the pellet (PLTs) was suspended in thrombin in Tyrode's buffer (0.1U/mL) to induce activation and incubated at 37°C for 1 h. The solution was then centrifuged at 3000 × g for 10 min at 24 ± 3°C to remove PLTs and the supernatant (PMPs) was centrifuged at 20,000 × g for 90 min at 18°C. The PMPs pellet was resuspended in platelet additive solution (PAS) and stored at −80°C. PMPs were thawed at 37°C then incubated with 100 μM doxorubicin (DOX) in PAS at 37°C for 1 h. The

supernatant was centrifuged at 20,000 × g for 90 min at 18°C. The pellet of PMPs loaded with DOX (PMPDs) was resuspended in PAS. The sizes and the concentrations of PMPs and PMPDs were measured using a nanoparticle tracking analysis (NTA). Data were analysed using NTA software. Transportation of DOX from PMPDs to breast cancer cell lines was observed by deconvolution microscopy.
Results: NTA results revealed that the mean size of PMPDs (234.1 ± 48.01 nm) was slightly larger compared with that of PMPs (200.1 ± 57.71 nm) and that DOX incorporation did not influence the quantification of PMPs. The concentration of them was no significant difference. The size distributions and images of PMPs and PMPDs indicated the absence of aggregated PMPs associated with DOX loading. When incubated with MCF-7 and MDA-MB-231 cells, PMPDs transferred DOX to the nuclei of cancer cells within 30 min.
Summary/Conclusion: These results support the potential clinical use of PMPDs as novel cell-based “Trojan Horse” anti-cancer therapeutic strategy.
Funding: This study was supported by the Ministry of Science and Technology.

PT11.06

Design of an exosome-based drug delivery system transporting anticancer peptides for targeting breast metastases in the brain

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Introduction: The treatment of breast cancer brain metastases can be addressed with the effective delivery of anti-tumoural drugs into the brain. The development of a drug delivery system (DDS) that can physiologically match the cell membrane, decrease the development of immune responses and that crosses biological barriers is significantly valuable for treating metastatic breast cancer (MBC). When compared to other nanoparticle delivery vehicles, exosomes represent an interesting approach to conventional DDS. In the present work, exosomes from breast cells were isolated and biophysically characterized. In addition, their interaction with anticancer peptides (ACPs) was unravelled envisioning the design of a DDS for MBC.
Methods: Exosomes from breast cell lines were isolated using a commercially available kit and biophysically characterized with transmission electron microscopy (TEM), atomic force microscopy (AFM), flow cytometry, Western Blot and dynamic light scattering. The interaction of PvD1 and vCPP2319 ACPs with the breast cells and respective exosomes was also followed with surface plasmon resonance (SPR) as to detail peptide’s binding to the different exosomes.
Results: Results suggests an intracellular target for vCPP2319 cytotoxic activity on breast cancer cells. The binding of the peptides to both membranes of human cells and exosomes results in cell death and in strong binding, respectively, pointing to the potential ability of these breast exosomes in transporting ACPs, which in turn are highly effective towards tumour cells.
Summary/Conclusion: Even though more studies are currently in development, the combination of potential ACPs with human-derived exosomes are shown as a potential source for a highly selective and effective DDS aiming to attack breast tumour cells located in the brain.
Funding: Fundação para a Ciência e a Tecnologia (FCT I.P., Portugal) is acknowledged for funding – PTDC/BBBBQB/1693/2014. F. O., J. S. and T. F. acknowledge FCT I.P., Portugal for fellowships PD/BD/135046/2017, PD/BD/114177/2016 and SFRH/BD/5283/2013, respectively. Marie Skłodowska-Curie Research and Innovation Staff Exchange (RISE) is acknowledged for funding: call H2020-MCA-RISE-2014, Grant agreement 644, 167, 2015–2019.

PT11.07

Embryonic stem cells-derived exosomes endowed with targeting properties as chemotherapeutics delivery vehicles for glioblastoma therapy

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Introduction: Glioma treatment is severely hindered by blood brain barrier (BBB) which leads to very limited on-target activity of therapeutic agents. Exosomes are nanosized extracellular vesicles with efficient BBB penetration ability and presents a promising drug carrier for glioma treatment. However, several reports have demonstrated that injected exosomes mainly distribute in liver and spleen rather than brain. In this study, we find embryonic stem cell derived exosomes (ES-Exos) show broad spectrum anti-tumour ability including glioma, and thus we further use ES-Exos as paclitaxel (PTX) carrier and modify them with tumour targeting ligand cRGD.

Methods: CCK-8 analysis and flow cell analysis were used to test the anti-tumour ability of ES-Exos. cRGD was incorporated onto the surface of ES-Exos by post-insertion methods with cRGD-DSPE-PEG2000 (cRGD-Exos), and PTX was loaded into cRGD-Exos by co-incubation to get cRGD-Exos-PTX. In situ glioma model of mice was built by injecting glioma cells in brain. *In vivo* imaging was used to test the biodistribution of cRGD-Exos-PTX. Further, subcutaneous tumour of mice was also built to evaluate the anti-tumour ability of ES-Exos and cRGD-Exos-PTX.
Results: Our results showed that ES-Exos could inhibit tumour cell proliferation of broad spectrum, including U87, U251, A549, HCC, HepG2, B16, MDA-MB-231 and DU145. Flow cell analysis showed that ES-Exos induced tumour cell apoptosis. Furthermore, after cRGD modification, cRGD-Exos showed enhanced tumour cell uptake compared with ES-Exos. And *in vivo* imaging analysis demonstrated that more cRGD-Exos distributed in glioma site in mice brain. And mice with in situ glioma treated with cRGD-Exos-PTX lived more longer than the group treated with Exos-PTX. Finally, cRGD-Exos-PTX showed the beat anti-tumour ability in subcutaneous tumour model.
Summary/Conclusion: In this study, we demonstrate that ES-Exos is antineoplastic, and their tumour site distribution is enhanced by cRGD modification. cRGD-Exos-PTX is an efficient therapeutic agent for glioma treatment.
Funding: NSFC Project No. 81671209 and No. 81471243.

PT11.08

Exosome as a vehicle for delivery of membrane protein therapeutics, PH20, for enhanced tumour penetration and antitumor efficacy

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Introduction: As biochemical and functional studies of membrane protein remain a challenge, there is growing interest in the application of nanotechnology to solve the difficulties of developing membrane protein therapeutics. Exosome, composed of lipid bilayer enclosed nanosized extracellular vesicles, is a successful platform for providing a native membrane composition.
Methods: Exosome Preparation and Characterization – DLS, western blot, TEM Enzymatic Activity Assay *in vitro* and *in vivo* HA Depletion Analysis Tumour Blood Flow Biodistribution Imaging of Dox Fluorescence Distribution in Tumours Evaluation of Anti-tumour Effect in Mouse Model.
Results: This study reports an enzymatic exosome, which harbours native PH20 hyaluronidase (Exo-PH20), which is able to penetrate deeply into tumour foci via hyaluronan degradation, allowing tumour growth inhibition and increased T cell infiltration into the tumour. This exosome-based strategy is developed to overcome the immunosuppressive and anticancer therapy-resistant tumour microenvironment, which is characterized by an overly accumulated extracellular matrix. Notably, this engineered exosome with the native glycosylphosphatidylinositol-anchored form of hyaluronidase has a higher enzymatic activity than a truncated form of the recombinant protein. In addition, the exosome-mediated codelivery of PH20 hyaluronidase and a chemotherapeutic (doxorubicin) efficiently inhibits tumour growth. This exosome is designed to degrade hyaluronan, thereby augmenting nanoparticle penetration and drug diffusion.
Summary/Conclusion: Here, we developed the engineered exosome that facilitates its own penetration into the HA-containing tumour ECM. Enabling chemical drugs, nanoparticles, and immune cells to penetrate deeply into tumour foci is a challenging goal of studies aimed at achieving antitumor therapeutic efficacy. The exosome-triggered infiltration of cytotoxic T cells into tumour tissues, which was observed in the present work, could induce an adaptive immune response to help combat cancer. Moreover, we provide a general strategy that may be used to decorate exosomal surfaces with natural-state membrane-bound proteins.

PT11.09

Surface engineering of exosomes to block HIV infection

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Introduction: While lifelong antiretroviral therapy has dramatically reduced the morbidity and mortality of HIV infection, treated individuals still experience immune dysregulation and chronic inflammation, driving interest in alternative therapeutic and curative strategies. Exosomes, extracellular membrane vesicles 30–100 nm in size, have shown promise as engineerable therapeutic agents for a broad range of diseases. We aimed to engineer exosomes with the capacity to block HIV infection as a novel antiviral approach.
Methods: Exosomes were isolated from 1 mL of healthy donor plasma using polymer-based precipitation and column purification. Nanoparticle tracking

analysis was used to determine the abundance and size of particles. Exosomes were quantified by fluorometer, and 200 μg protein equivalents were decorated with single-chain variable fragment (scFv)-C1C2 fusion proteins with complementarity determining regions targeting the HIV envelope protein. The HIV-1 NL4-3 EGFP reporter virus was incubated with decorated exosomes for 2 h at 1:1, 1:2 and 1:4 ratios. Virus was incubated with no exosomes, undecorated exosomes, or anti-PD-1 scFv-decorated exosomes as negative controls. Jurkat E6.1 cells and primary human CD4+T cells were infected with virus-exosome preparations via spinoculation, and GFP fluorescence was measured by flow cytometry to determine infection levels after 72 h.

Results: Our engineered anti-HIV scFv-decorated exosomes significantly inhibited HIV infection in Jurkat cells with respect to all negative controls ($n = 3$; $p < 0.05$, paired *t*-test). Anti-HIV scFv-decorated exosomes potently inhibited HIV infection in primary human CD4 + T cells ($n = 2$ donors) in a dose-dependent manner, suppressing up to 87% of infection in the absence of toxicity.

Summary/Conclusion: Engineering exosomes *ex vivo* represents a promising therapeutic approach for HIV infection. Future work will test the capacity of our designer exosomes to inhibit HIV replication *in vivo* in humanized mouse models. Beyond viral suppression, we will determine if designer exosomes can accelerate the clearance of HIV latently-infected cells, the main obstacle to a cure for HIV infection.

Funding: NIH P01AI131374 and R01GM117901

PT11.10

Exosome-mediated RNAi of PAK4 prolongs survival of pancreatic cancer mouse model after loco-regional treatment

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Introduction: Pancreatic cancer (PC) remains one of the most aggressive and devastating malignancies, predominantly due to the absence of a valid biomarker for diagnosis and limited therapeutic options for advanced disease. Exosomes (Exo) as cell-derived vesicles are widely used as natural nanocarriers for drug delivery. P21-activated kinase 4 (PAK4) is oncogenic when over-expressed, promoting cell survival, migration and anchorage-independent growth. In this study, we validate PAK4 as a therapeutic target in an *in vivo* PC tumour mouse model using Exo nanocarriers following intra-tumoural administration.

Methods: PC derived Exo were firstly isolated by ultracentrifugation on sucrose cushion and characterized for their surface marker expression, size, number, purity and shape. siRNA was encapsulated into Exo via electroporation and dual uptake of Exo and siRNA was investigated by flow cytometry and confocal microscopy. *In vitro* siPAK4 silencing in PC cells was assessed by western blotting, flow cytometry, and *in vitro* scratch assay. *In vivo* efficacy (tumour growth delay and mouse survival) of siPAK4 was evaluated in PC bearing NSG mouse model. *Ex vivo* tumours were examined using Haematoxylin and eosin (H&E) staining and immunohistochemistry.

Results: High quality PC derived PANC-1 Exo were obtained. siRNA was incorporated in Exo with 16.5% loading efficiency. Exo and siRNA co-localization in cells was confirmed by *in vitro* imaging. PAK4 knock-down was successful at 30 nm Exo-siPAK4 at 24 h post-incubation *in vitro*. Intra-tumoral administration of Exo-siPAK4 (1 μg siPAK4 and 7.7 × 1011 Exo, each dose, two doses) reduced PC tumour growth and enhanced mice survival ($p < 0.001$), with minimal toxicity observed compared to polyethylenimine (PEI) used as a commercial transfection reagent. H&E staining of tumours showed significant tissue apoptosis in siPAK4 treated groups.

Summary/Conclusion: PAK4 interference prolongs survival of PC bearing mice suggesting its candidacy as a new therapeutic target in PC. PANC-1 Exo demonstrated comparable efficacy but safer profile than PEI as *in vivo* RNAi transfection reagent.

Funding: The K. C. Wong Education Foundation and The Marie Skłodowska-Curie actions, "Horizon 2020" project, EU (H2020-MSCA-IF-2016).

PT12: EV Based Therapeutics
Chairs: Mario Gimona; Saara Laitinen
Location: Level 3, Hall A

15:30–16:30

PT12.02

Exosomes from adipocyte-derived stem cells reduce the oxidative stress through the mitochondrial uncoupling in pantothenate kinase 2 mutation *in vitro* models

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Introduction: The exosome is a promising novel therapy for human diseases. Exosomes-derived from mesenchymal stem cell is thought to contain plenty of unique microRNA, which is specific for boosting cellular repair and regeneration. Neurodegenerative diseases are characterized by the neuronal pre-mature apoptosis and the lack of the ability of regeneration. It is hypothesized that the supplement of exosomes-derived from the stem cells could activate the expression of neuroprotective gene/protein expression, resume the impaired cellular function and reverse the degenerative process.
Methods: Patient with pantothenate kinase 2 (PANK2) mutation-related neurodegeneration with brain iron accumulation was recruited and the leukocytes were immortalized to establish the *in vitro* models. The adipose-derived stem cell (ADSC) was obtained from the healthy donors and the exosomes were isolated from the culture medium at confluent.
Results: The PANK2 mutation resulted in the elevated oxidative stress and depolarization of mitochondria. Exosome treatment (5 μg of exosome suspension upon $3*10^6$ leukocytes) for 24 h up-regulated the protein level of mitochondrial uncoupling protein 2 and 3, as well as boosted the mitochondrial biogenesis, assessed by the protein level of PGC-1α and TOMM20. Those proteins were undatable in the exosomes themselves. Mitochondrial uncoupling proteins are responsible for the dissipating of mitochondrial membrane potential and down-regulation of the oxidative phosphorylation from respiration, which is the major source of free radical and oxidative stress. Exosome treatment for 24 h led to the obvious mitochondrial depolarization, assessed by JC-1 and further reduction of the oxidative stress, assessed by the H2DCFDA.
Summary/Conclusion: The exosomes from ADSC were able to reduce the oxidative stress through manipulating mitochondrial functions. The effect is speculated to achieve by the modulation of genetic expression in the recipient cells.
Funding: Minister of Science and Technology, Taiwan (MOST 107–2314-B-038 –086 -MY2)

PT12.03

Extracellular vesicle-secretion system based on agarose gel encapsulation of cells for cell therapy

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Introduction: Extracellular vesicles (exosomes, EVs, 30 ~ 200 nm in diameter) are released from various types of cells. Because EVs carry functional molecules such as e.g. microRNAs and enzymes, EVs play crucial roles in cell-to-cell communication. On the other hand, EVs have pharmaceutical advantages as carriers for intracellular delivery of therapeutic molecules, including, e.g. encapsulation of natural and/or artificial therapeutic/diagnostic molecules, controlled immunoreaction, effective usage of cell-to-cell communication routes, infinite secretion and expression of functional proteins in EV membranes. We are currently developing cell encapsulated gel system for secretion of functional EVs in cell therapy. In this research, agarose gels, which has been widely used in cell culture and chamber, is used for encapsulation of cells that secrete functional EVs from the gels. We here demonstrate our methods for cell encapsulation in the gels and cellular uptake efficacy of secreted EVs from the gels.
Methods: CD63 (EV marker protein)-GFP stably expressing HeLa cells were encapsulated using collagen and agarose gels. Secreted EVs from the gel system were separated using ultracentrifuge and analysed by western blotting, zeta potential, DLS and electron microscope (TEM). Cellular uptake of secreted EVs from the gels was observed using confocal laser scanning microscope.

Results: In the experimental optimization for encapsulation of cells in gels, we successfully attained CD63-GFP stably expressing HeLa cells-encapsulated agarose (1.5%) gels (e.g. 5 × 104 cells can be encapsulated in approx. 2 mm × 25 mm × 25 mm sheet-like gel). DLS analysis showed 30 ~ 100 nm EVs secreted from the gels, and zeta potential of the EVs was average −17 mV. Western blotting confirmed expression of exosomal marker proteins (e.g. CD63 and CD81). A431 cells (human epidemoid carcinoma) were cultured with the CD63-GFP stably expressing HeLa cells-encapsulated agarose gels for 24 h, and efficient cellular uptake of secreted EVs (CD63-GFP-EVs) from the gels were observed using confocal laser scanning microscope.
Summary/Conclusion: Although we have to conduct further optimization in this system as next step to obtain sophisticated methodology, these experimental techniques and findings will contribute to development for cell therapy based on EVs as basic studies.

PT12.04

Extracellular vesicles from endothelial progenitor cells improve outcomes of the lipopolysaccharide-induced acute lung injury

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Introduction: The acute respiratory distress syndrome is characterized by disruption of the alveolar-capillary barrier resulting in accumulation of proteinaceous oedema and increased inflammatory cells in the alveolar space. We previously found that extracellular vesicles (EVs) from endothelial progenitor cells (EPCs) prevent endothelial dysfunction and lung injury in sepsis due to their encapsulation of miRNA-126. However, the effects of EPC EVs in acute lung injury (ALI) remains unknown.
Methods: To determine if EPC EVs would have beneficial effects in ALI, intratracheal administration of lipopolysaccharide (LPS) was used to induce ALI in mice. Lung permeability, inflammation and the role of miRNA-126 in alveolar epithelial barrier function were examined.
Results: The intratracheal administration of EPC EVs reduced lung injury following LPS-induced ALI at 24 and 48 h. Compared to placebo, intratracheal administration of EPC EVs significantly reduced the cell number, protein concentration and cytokines/chemokines in the bronchoalveolar lavage fluid, indicating a reduction in permeability and inflammation. Further, EPC EVs reduced myeloperoxidase activity and reduced the lung injury score, demonstrating protection against lung injury. Murine fibroblast (NIH3T3) EVs, which do not contain abundant miRNA-126, did not provide these beneficial effects. In human small airway epithelial cells, we found that overexpression of miRNA-126-3p can target phosphoinositide-3-kinase regulatory subunit 2, while overexpression of miRNA-126-5p inhibits the inflammatory cytokine HMGB1 and permeability factor VEGFα. Interestingly, both miR-126-3p and 5p increase the expression of tight junction proteins suggesting a potential mechanism by which miRNA-126 may mitigate LPS-induced lung injury.
Summary/Conclusion: Our data demonstrated that human EPC EVs are beneficial in LPS-induced ALI mice, in part through the delivery of miRNA-126 into the injured alveolus.
Funding: 1R01GM113995 (HF), 1R01GM130653 (HF), 1K23HL135263-01A1 (AG), UL1TR001451 (PVH)

PT12.05

Hsa_circ_0000077-overexpressing extracellular vesicle: a new tool to prevent cartilage degeneration

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Introduction: Trauma and degeneration of articular cartilage (AC) could trigger the morbidity of one of the leading disabling disease, osteoarthritis (OA). One of the most difficult issues in treatment is the poor self-healing ability of AC. Extracellular vesicle (EV) transplantation has received more and more attention as potential cell-free therapeutic approaches to promote tissue healing. In our preliminary study, we found that decreased expression of hsa_circ_0000077 (circ77) was closely related to OA. And circ77-overexpression in chondrocytes can prevent the chondrocyte degeneration. In this study, EVs derived from circ77-overexpressing synovium mesenchymal stem cells (SMSC-77-EVs) were used to promote cartilage regeneration.
Methods: CCK-8, qPCR and western blotting (WB) were used to investigate the biological functions of SMSC-77-EVs on the proliferation and cartilage regeneration. Furthermore, interleukin 1β (IL-1β) were used to simulate the inflammatory conditions of OA, and then, the protective effects of SMSC-77-EVs were confirmed by CCK-8, qPCR and WB.
Results: CCK-8 assay confirmed that SMSC-77-EVs enhanced the proliferation of chondrocytes, compared with normal control and EVs derived from synovium mesenchymal stem cells which were transfected by empty vectors (SMSC-Empty-EVs). WB and qPCR assays confirmed that SMSC-77-EVs enhanced the

expression levels of cartilage related proteins including Type II collagen (Col-II), aggrecan (ACAN) and SOX9, compared with normal control and SMSC-Empty-EVs. IL-1β significantly inhibited the proliferation and cartilage regeneration-related proteins (Col-II, ACAN and SOX9). SMSC-77-EVs could observably restrain the harmful effects of IL-1β, while SMSC-Empty-EVs showed limited ability.

Summary/Conclusion: These findings suggest that the novel SMSC-77-EVs provides the preferable function in promoting the repair of cartilage damage. The use of SMSC-77-EVs would represent a development trend of cell-free therapies, using engineered EVs (or modularized EVs), for promoting cartilage regeneration.

Funding: The National Natural Science Foundation of China [Nos. 81871834, 81802226 and 81301589], and Shanghai Jiao Tong University K.C.Wong Medical Fellowship Fund supported this work.

PT12.06

Lymphangiogenesis induced by exosomes derived from adipose-derived mesenchymal stem cells

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Introduction: Lymphedema is chronic oedema of limbs caused by the accumulation of lymphatic fluid and characterized by a progressive disorder of the smooth muscle cells of the lymphatic channels. Transplantation of adipose-derived mesenchymal stem cells (ADSCs) has been reported to improve the severity of lymphedema, however, the detailed mechanism has not been elucidated yet. Extracellular vesicles(EVs) derived from mesenchymal stem cells have been reported to have functions such as cancer development, angiogenesis, suppression of inflammation, regeneration of damaged organs and treatment of degenerative disease. ADSCs are thought to be promising source of regenerative medicine, and EVs derived from ADSCs are thought to have similar effects as well. Here, we analysed lymphangiogenesis induced by EVs derived from ADSCs for treatment of chronic lymphedema.

Methods: EVs derived from ADSCs were isolated by ultracentrifugation. The effect of EVs to lymphatic endothelial cells (LECs) were analysed in proliferation assay, migration assay and tube formation assay. Gene expression analyses were also performed by qRT-PCR. LECs were treated with PBS as control, VEGF-C(10 ng/ml) and ADSC-EVs(100 μg/ml) one time in each assay. The incubation time was 48 h in proliferation assay, 16 h in migration assay, 8 h in tube formation assay and 12 and 24 h in qRT-PCR.

Results: ADSC-EVs group showed almost one point five to twice increase of proliferation, migration and tube formation function compared to PBS group. Furthermore, gene expressions for lymphatic markers such as VEGFR-3, Lyve-1, Podoplanin, Prox-1 were also shown almost two to five times increase in the ADSC-EVs group.

Summary/Conclusion: The present study showed lymphangiogenic effects of EVs derived from ADSCs, which lead to new treatment options for chronic lymphedema. Further studies are needed to elucidate what kind of molecular in ADSC-EVs works in LEC. In vivo studies using mouse lymphedema model are also needed to confirm the biological function of ADSC-EVs. EVs for cell free therapy are less potential risk compared to stem cell transplantation and could be promising tool for patients suffering from lymphedema.

Funding: JSPS Kakenhi; Takeda Science Foundation.

PT12.07

Embryonic stem cell-derived extracellular vesicle-mimetic nanovesicles rescue erectile function by enhancing penile neurovascular regeneration in the streptozotocin-induced diabetic mouse

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Introduction: Extracellular vesicles (EV)-mimetic nanovesicles (NVs) contains a variety of protein, mRNA and miRNA and is known to play an important role in intercellular communication as a bio-nanoparticle with a diameter of 40 to 100 nm. Recent studies have demonstrated the therapeutic potential of EV-mimetic NVs in a variety of animal models for cardiovascular diseases and neuropathies. The aim of this study was to investigate effectiveness of embryonic stem cell (ESC)-derived EV-mimetic NVs in restoring erectile function in diabetic mice.

Methods: Diabetes was induced by intraperitoneal injection of streptozotocin into 8-week-old C57BL/6 male mice. At 8 weeks after the induction of diabetes, the animals were distributed into 7 groups: control non-diabetic mice and diabetic mice receiving two successive intracavernous injections of HEPES-buffered saline (HBS, days −3 and 0; 20 μL) or ESC-

derived EV-mimetic NVs (ESC-NVs, days −3 and 0; 0.1 μg, 0.5 μg, 1 μg, 2 μg, or 5 μg in 20 μL of HBS, respectively). Two week after treatment, we measured erectile function by electrical stimulation of the cavernous nerve. The penis was then harvested for histological and biochemical studies. We also examined the effects of ESC-Exo in primary cultured mouse cavernous endothelial cells (MCEC) and pericytes (MCP) in vitro; and in cultured aortic ring and major pelvic ganglion (MPG) *ex vivo*.

Results: Intracavernous injections of ESC-NVs significantly improved erectile function in diabetic mice, which reached up to 90% of control values. ESC-NVs induced significant restoration of cavernous contents of endothelial cells, smooth muscle cells, pericytes, and neuronal cells in diabetic condition. Moreover, ESC-NVs promoted micro-vascular sprouting from aortic ring and accelerated tube formation in primary cultured MCEC and MCP mono-culture or co-culture system *in vitro*.

Summary/Conclusion: ESC-NVs successfully restored erectile function through enhanced cavernous angiogenesis and neural regeneration in diabetic mice. It will be a better strategy to use ESC-NVs than ESCs for the treatment of retractable erectile dysfunction although it remains to be solved for future clinical application of ESCs.

PT12.08

Human adipose tissue-derived mesenchymal stem cell exosomes for the treatment of liver fibrosis

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Introduction: Liver fibrosis, often leading to liver cirrhosis and cancer, is a challenging health issue for which there are no effective medicines. Conventional therapies are merely symptomatic treatment. Hence, there have been lots of efforts to develop better therapeutic strategies. Cell therapy based on mesenchymal stem cells (MSCs) is one of the attractive options. MSCs hold immunomodulatory properties and multipotency. However, cell transplantation has limitations such as teratoma formation and low cell viability *in vivo* condition. In order to overcome these limitations, we suggest MSCs derived exosome-based therapy for treating liver fibrosis.

Methods: Exosome-derived from ADSCs (A-exo) was purified using a tangential flow filtration system. The physical characteristics of A-exo were investigated using DLS, TEM and NTA. The protein concentration of A-exo was determined by BCA assay. The effect of A-Exo on the expression level of α-SMA was evaluated by IF analysis. Mice were received thioacetamide intraperitoneally. Fluorescently labelled A-exo was administered to mice and whole-body fluorescence was observed in order to evaluate the *in vivo* distribution. The therapeutic efficacy of A-exo was determined by measuring the level of ALT, ALP, TBIL and TP in blood of mice. A-exo was injected intravenously three times and blood was collected after final injection.

Results: When hepatic stellate cells were activated with TGF-β1, the expression level of α-SMA was significantly increased. While, the level was remarkably decreased depending on the treatment concentration of A-Exo. A-exo treatment significantly decreased expression mRNA of pro-fibrogenic marker: α-SMA, Collagen I and MMP-2. After systemic administration of exosome, a substantial accumulation of A-Exo at liver was observed in both the normal and mice model of liver fibrosis. Furthermore, liver function of A-exo treated group was restored to normal. These results showed A-exo had the high therapeutic efficacy.

Summary/Conclusion: In this study, we investigate the potential of stem cell-derived exosome as the new therapeutic approach for liver fibrosis treatment. A-exo has similar bioactive capacity to its origin cell, mesenchymal stem cell. The beneficial effect of A-exo was confirmed *in vitro* and *in vivo* tests. The superior therapeutic efficacy was displayed in A-exo treated mouse group.

PT12.09

HucMSC exosome confer protection against ultraviolet radiation induced acute photodamage via modulation of SIRT1 pathway

Peipei Wu[a], Hui Qian[b] and Wen Rong Xu[c]

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Introduction: Exosomes are nano-sized membrane vesicles secreted by most cells, including human umbilical cord mesenchymal stem cells (hucMSC). hucMSC derived exosomes (hucMSC-ex) have been reported to significantly facilitate skin regeneration, resembling the effect of parental cells. However, the role of hucMSC-ex in ultraviolet radiation (UV) induced skin damage and the underlying mechanisms are largely unknown.

Methods: Herein, we examined the benefit of hucMSC-ex in a rat acute skin photodamage model.

Results: We found that the subcutaneous injection of hucMSC-ex (1 mg) elicited noted antioxidant and anti-

inflammatory effects against UV induced DNA damage and apoptosis *in vivo*. Further studies shown that sirtuin1(SIRT1) expression level in skin keratinocytes (HaCaT) decreased in a time- and dose-dependent manner under oxidative stress *in vitro*, however, the treatment of hucMSC-ex reverses this phenomenon. Activation of SIRT1 significantly attenuated UV and H_2O_2-induced cytotoxic damage by inhibiting oxidative stress and promoting autophagy activation. Furthermore, we also discovered that the cytoprotection function provided by hucMSC-ex carried 14–3-3ζ was potential associated with modulation of SIRT1 dependent antioxidant response.

Summary/Conclusion: Collectively, our findings indicated that hucMSC-ex is a new potential agent for preventing and/or treating UV-induced skin photodamage and ageing.

Funding: Zhenjiang Key Laboratory of Exosomes Foundation and Transformation Application High-tech Research, china: (ss2018003). National Natural Science Foundation of China: (BE2016717).

PT12.10

Anti-melanogenic effect screening for natural plant-derived exosome-like nanovesicles

Ruri Lee[a], Yeon Juhun[b], Kim Kimin[a], Min seo yun[c] and Kim Jung Ah[c]

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Introduction: Demand for whitening agents is increasing due to their anti-melanogenic effects by improving skin darkness and decreasing melanin production in the cosmetics industry. However, there have been side effects and high toxicity issue as well as poor skin penetration. Therefore, many researchers have focused on natural plants as an alternative chemo-therapeutics agent to avoid various side effects. Recently, it is known that exosome-like nanovesicles have biocompatibility and excellent drug delivery capacity. In this study, leaves and stems-derived exosome-like nanovesicles were isolated from *Dendropanax Morbifera* and we have found that inhibition of those nanovesicles on melanin products.

Methods: Exosome-like nanovesicles from leaves and stems were isolated and identified size using DLS and NTA. These shapes were observed by TEM. The anti-melanogenic effect was verified by evaluating the melanin content and tyrosinase activity on melanoma cell. Also, western blot was used to observe melanogenesis-related protein expression. In addition to, cellular melanin formation was confirmed using TEM. The human epidermal model was used to evaluate the inhibition of melanogenesis.

Results: The leaves and stems-derived exosome-like nanovesicles are able to suppress cellular melanin content melanoma cells. Also, melanogenesis protein expression was reduced with leaves- and stems-derived exosome-like nanovesicles. These results suggest that leaves- and stems-derived exosome-like nanovesicles of the *D. morbifera* could be a candidate of natural substances for anti-melanogenic agents.

Summary/Conclusion: The leaves and stems-derived exosome-like nanovesicles are able to suppress cellular melanin content melanoma cells. Also, tyrosinase activity and melanogenesis protein expression were reduced with leaves- and stems- derived exosome-like nanovesicles. These results suggest that leaves- and stems-derived exosome-like nanovesicles of the *D. morbifera* could be a candidate of natural substances for anti-melanogenic agents.

Funding: This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2016R1C1B2013345).

PT12.11

Stem cell extracellular vesicles as therapeutics for autoimmunity

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Introduction: Stem cells including mesenchymal stem cells (MSC) hold great potential in treating autoimmune disorders. However, their clinical translation has been hindered due to incomplete understanding of mechanisms of action (MOA) and potential safety concerns. Recent evidence revealed that some of the MSC MOA may be associated with extracellular vesicles (EV),

Methods: We investigated MSC derived exosomes in immune modulation in a multiple sclerosis experimental autoimmune encephalomyelitis (EAE) a mouse model in vivo as well as in T cell proliferation suppression and Treg induction in vitro.

Results: Our results indicated that that intravenous administration of exosomes produced by MSCs stimulated by IFNγ (IFNγ-Exo) (i) enhanced the mean clinical score of EAE mice compared to PBS control, (ii) home into the spinal cords and reduced demyelination, (iii) decreased neuroinflammation and (iv) upregulated the number of CD4+/CD25+/FOXP3+regulatory T

cells (Tregs). In addition, we found that IFNγ-Exo significantly reduced the proliferation of T-cells in vitro and reduced production of proinflammatory factors including IL-6, IL-17 and IL-22 while enhanced the production of Indoleamine 2,3-dixygenase (IDO), a key player in MSC-mediated immunosuppression.

Summary/Conclusion: Our findings suggest that stem cell derived EVs can serve as promising candidates in treating autoimmune and neurodegenerative diseases.

Funding: National Institute of Health (1DP2CA195763-01; #NS082174).

PT12.12

Biocompatible myxobacteria-derived outer membrane vesicles show inherent antibacterial activity against gram-negative and gram-positive microbes

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Introduction: Myxobacteria are gram-negative bacteria that live in the soil and can be found in different habitats and they have been explored for their capability of providing natural products with great potential for antimicrobial therapy. We investigated the antimicrobial activity of OMVs isolated from the myxobacterial strains *Cystobacter velatus* (Cbv34) and *Cystobacter ferrugineus* (Cbfe23) against *E. coli* DH5-alpha and *S. aureus* Newman. Cbv34 and Cbfe23 are producers of the antibacterial compound cystobactamid that we hypothesized to be naturally loaded into the OMVs they shed.

Methods: The OMVs were isolated by differential centrifugation and size-exclusion chromatography. Particle size, distribution, concentration and morphology were analysed by nanoparticle tracking analysis and cryo-transmission electron microscopy. Particle interaction and uptake into macrophages (THP-1) and lung alveolar epithelial cells (A549) was investigated by confocal laser scanning microscopy and flow cytometry. Cytotoxicity and viability assays were performed using PrestoBlue and lactate dehydrogenase assays. The antibacterial activity of the OMVs was assessed by overnight incubation with *E. coli* or *S. aureus*, followed by optical density measurement and CFU determination. The OMVs content was investigated by liquid chromatography coupled mass spectrometry.

Results: Both OMVs did not induce cytotoxicity or negatively influenced the viability of THP-1 and A549 cells even at concentrations of 10,000 OMVs/cell. Cbv34 and Cbfe23 OMVs showed a bactericidal activity against *E. coli* and *S. aureus*. The antibacterial effect of Cbv34 OMVs remained potent upon storage at 4°C for 4 weeks. The presence of cystobactamid in Cbv34 OMVs was confirmed by MS. Using flow cytometry and labelled OMVs, we observed 60% positive cells for Cbv34 OMVs and 40% positive cells for Cbfe23 OMVs with THP-1, and 50% positive A549 cells for Cbv34 OMVs after 4 h of incubation.

Summary/Conclusion: The biocompatibility, inherent bacterial activity and uptake into mammalian cells may be promising for the treatment of infections, especially those induced by intracellular *S. aureus*. However, their mechanism of action needs further investigation.

Funding: This work was supported by the Federal Ministry for Research and Education through the NanoMatFutur programme.

PT12.13

Placental MSCs and their exosomes as vehicles for the Na/I symporter (hNIS): a new theragnostic agent

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Introduction: The Na > I symporter gene (hNIS) is expressed in the thyroid and allows the accumulation of iodine from the diet, to form T3 and T4 hormones. Moreover, it is widely used (i) as a reporter gene for molecular imaging (when the positron emitter isotope is I^{124} for PET or Tc^{99} for SPECT) or (ii) as a therapeutic gene for cancer therapy, mediated by the accumulation of 1131. An unresolved challenge is how to direct this gene specifically to the tumoral area.

Previously, our group demonstrated the migratory capacity of placental mesenchymal stem cells (MSCs), carrying an adenovirus expressing hNIS to tumours, with good results as a theragnostic tool. However, as hNIS is expressed at the placental tissue (because it transfers iodine to the foetus from the maternal blood), in this work we decided to study whether placental MSCs and their derivatives (exosomes) (1) express hNIS endogenously and therefore transfers the imaging and therapeutic potentials when administered with radioactive iodine (2) are capable to reach the tumoral areas when they are intravenously injected

due to the tumoral tissues extravasation.The aim of this research was to develop a new anti-tumoural therapy by the combination of the advantages of both NIS and the human placental MSCs (hPMSCs).

Methods: Here, we used two approaches using the endogenous hNIS expression, first in hPMSCs but also on its exosomes. For both cases, we determined in vitro NIS location and functionality but also we followed those vectors by SPECT CT and studied their antitumoral effect after radioactive iodine injection.

Results: We proved that human placenta MSCs and their exosomes have endogenous expression of NIS, migrate specifically to the tumour and their endogenous expression of NIS is enough to visualized in vivo cells and exosomes accumulation and to see significant therapeutic effect on cancer treatment with 131I.

Summary/Conclusion: Our findings highlight the possibility to use endogenous expression of NIS as therapy and opening a wide range of new possibilities to treat cancer.

Funding: This work has been funded by Universidad Francisco de Vitoria, Instituto de Salud Carlos lll and Instituto Aragonés de Ciencias de la Salud

LBT01: Late Breaking- Technological advances
Chairs: M. Selim Unlu, Olga Shatnyeva
Location: Level 3, Hall A

15:30–16:30

LBT01.01=OWP1.09

Coagulation influences properties of extracellular vesicles isolated from autologous blood derived products

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Introduction: Platelet rich plasma (PRP) is the most commonly used blood derivative in clinics due to its high concentration of platelets and perceived high growth factor levels. Drawbacks of using PRP are discrepancies among preparation protocols and the presence of cells (platelets, leuxcocytes) which can evoke cellular processes (e.g. inflammation) when injected into the host. One possibility is to isolate only the active components of blood derivatives which may overcome this problem. In the current study we focused on extracellular vesicles (EVs) isolated from two autologous blood derivatives, PRP and hyperacute serum and investigated whether the clotting cascade influences EV properties.

Methods: EVs were isolated from citrate-anticoagulated PRP (CPRP) and hyperacute serum using differential ultracentrifugation followed by a size exclusion chromatography. Particle concentration and size were determined by nanoparticle tracking analysis (NTA). Cryo-electronmicroscopy was performed to visualize isolated EVs. Expression of miRNAs transported within EVs as well as in their respective input material was analysed by qPCR.

Results: NTA revealed higher particle concentrations and bigger sized EVs within CPRP compared to hyperacute serum. These findings were confirmed by cryo-electronmicroscopy. Profound differences were detected regarding miRNA expresion between the two blood derivatives. 126 miRNAs were identified which were expressed both in input material as well as in the corresponding EVs. The correlation between miRNAs in EVs and input material was higher in CPRP compared to hyperacute serum meaning that in hyperacute serum miRNAs were identified which were higher expressed in EVs than in the corresponding input material.

Summary/conclusion: EVs from autologous blood products represent a novel and cell free regeneration approach. We observed that the clotting cascade (plasma versus serum) has an influence on concentration, size and miRNA expression patterns of EVs. These differences might have an impact on the biological mode of action of blood derived products used in clinics.

Funding: Financial support was received from the European Fund for Regional Development (EFRE) and the Science Fund of Lower Austria. miRNA expression analysis was performed by TAmiRNA GmbH. Cryo-electronmicroscopy was conducted at the Core Facility of the Vienna Bio-Center.

LBT01.02=OWP1.11

Ev-avogadro project: towards a liposomal concentration standard for extracellular vesicle research

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Introduction: There is an unmet need for standardization of concentration measurements in the field of extracellular vesicles (EVs). Liposomes may serve an ideal reference system for EVs, but the determination of the number concentration of liposomes from first principles was not attempted so far. Inspired by the International Avogadro project, we aimed to determine the concentration of liposomes with well-defined size and composition via counting the number of phospholipid molecules in these “nanospheres”.

Methods: Liposomes composed of phosphocholine and phosphoglycerol were prepared by the extrusion method. Wide-angle X-ray scattering (WAXS) was used to determine the area-per-lipid value. The size distribution of the liposomes was determined by microfluidic resistive pulse sensing (MRPS) and freeze-fracture combined TEM. Small-angle X-ray scattering (SAXS), differential scanning calorimetry (DSC), and infrared spectroscopy (IR) were used to prove the unilamellarity, the ideal miscibility of the lipids and the

ordered packing of the hydrocarbon chains of the lipids, respectively. Concentration of the lipids was determined by liquid chromatography–mass spectrometry (LC-MS).

Results: The prepared liposomes proved to be unilamellar with narrow size distribution (83 nm avg.), as obtained by MRPS and TEM. DSC and IR measurements confirmed that the phospholipid bilayer of these liposomes is in the liquid-ordered phase, hence the area-per-lipid of 0.41 nm2 was determined from WAXS measurements. Using the concentration of phospholipids from LC-MS measurements, the number concentration of liposomes was determined (8E+13 1/mL).

Summary/conclusion: Liposomes containing saturated phospholipids are in the liquid-ordered phase, which can be utilized to determine the area-per-lipid using WAXS. This value, together with the independently determined size, and lipid concentration can be used to calculate the number concentration of liposomes. As the light scattering properties of liposomes matches that of EVs, liposome based standards for optical measurements of EVs can be obtained with the presented techniques.

Funding: This work was supported under grant numbers PD 121326 and NVKP_16-1-2016-0007 by NKFIH (Hungary). ZV was supported by the János Bolyai Research Fellowship.

LBT01.03

Standards for EV research

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Introduction: Progress in understanding the origins, composition, and effects of extracellular vesicles (EVs) depends on the reproducibility and rigor of experimental results. Standards can improve experimental rigor and reproducibility and promote data sharing. To address the needs for standards for single EV analysis, we have developed a set of standardized vesicle preparations and characterized them with respect to number, size, and cargo using a suite of single EV characterizations methods.

Methods: We prepared synthetic lipid vesicles with a lipid composition approximating that of a mammalian cell plasma membrane and extruded through a nucleopore membrane (100 nm mean pore diameter). We prepared cell-derived EVs from washed red blood cells (RBCs) and platelets (PLTs), and from cultured cell lines using centrifugation and ultrafiltration. EV size and number were evaluated using microfluidic resistive pulse spectroscopy (MRPS), nanoparticle tracking analysis (NTA), cryo-electron microscopy (cryo-EM), conventional light scatter-based flow cytometry (FC), and fluorescence-based vesicle flow cytometry (VFC). EV surface markers were measured using VFC with well-characterized fluorescence-labelled antibodies and calibrated using fluorescence intensity and antibody binding standards.

Results: Cell-derived EVs are stable for months at −80C and weeks at 4C, as assessed by measurement of number, size distribution, and surface markers. RBC EVs had a median diameter of 115 nm and expressed a median of ~2700 anti-CD235ab binding sites per EV, while PLT EVs had a median diameter of 145 nm and expressed a median of ~1200 anti-CD41 binding sites per EV.

Summary/conclusion: EV standards that are well characterized at the single EV level in terms of number, size, and molecular cargo can facilitate assay validation, sharing of data and results between labs, and support the development of new analysis technologies with improved sensitivity, resolution, and throughput.

Funding: Supported by the US National Institutes of Health.

LBT01.04

Cell-specific EV tetraspanin expression

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Introduction: Tetraspanins (TSs) are integral membrane proteins present on plasma and internal membranes and are thought to affect membrane organization and function. Tetraspanins can also be found in extracellular vesicles released from cells and have been considered canonical EV markers. To gain insight into the significance of TS expression on EVs, we used single vesicle flow cytometry (VFC) to measure the TS expression on individual EVs from different cell sources.

Methods: EVs were prepared from 10 different cell lines cultured in seru-free media and enriched by ultracentrifugation or ultrafiltration. EVs from washed red blood cells (RBCs) and platelets (PLTs) by were isolated by centrifugation, and characterized by nanoparticle tracking analysis (NTA), microfluidic resistive pulse spectroscopy (MRPS), cryo-electron microscopy (cryo-EM), and vesicle flow cytometry (VFC). TS

expression was measured using a panel of phycoerythrin-conjugated monoclonal antibodies against CD9, CD63, CD81, CD82, CD151, CD53 and CD231. The fluorescence scale was calibrated using intensity standard meads and expressed as PE MESF (mean equivalent soluble fluorochromes).

Results: The "canonical" TS EV markers CD9, CD63, and CD81 were expressed on EVs from all cells except RBCs, which expressed detectable amounts (LOD ~25 MESF) of no TS, but the relative and absolute amounts varied drastically from cells which expressed primarily CD9 molecules on EVs (PLT and A431), to those that expressed predominantly CD63 (MCF7, U87) to those that expressed predominately CD81 (293T, iPSC-derived neurons). Moreover, EVs from most cells expressed some level of CD151, while CD82 was detected on EVs from A431 and U87MG cells.

Summary/conclusion: Tetraspanins appear to be involved in many different cellular processes and their specific roles in EV-related physiology is not understood. Single vesicle analysis of TS expression using VFC reveals the diversity in TS expression and abundance on EVs from different cell types. Understanding the tetraspanin expression on EVs may provide information about the cellular origin of EVs, their effects on recipient cells, or both.

Funding: Supported by the US National Institutes of Health.

LBT01.05

Characterization of lipid profile of extracellular vesicles and lipoproteins in human plasma and serum

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Introduction: Extracellular vesicles (EVs) are lipid bilayer nano-vesicles existing in various biofluids, and regarded as valuable sources for biomarker. To data, the main target field of previous biomarker studies on EVs are proteome and transcriptome. Meanwhile, liquid chromatography coupled with high resolution mass spectrometry (LC-MS) has recently been employed to study comprehensive lipid profiles of *in vitro* EVs and their parental cells. However, lipid profile of EVs in biolfluids, especially blood specimens such as plasma and serum, has not been well-characterized. To use control data for EVs, we aimed to characterize lipid profile of EVs in human healthy plasma and serum, and to compare their lipid profile with that of other lipid-containing particles in blood, high density lipoproteins (HDL) and low/very low density lipoproteins (LDL/VLDL).

Methods: EVs, HDL and LDL/VLDL fraction were collected from 12 plasma or serum samples obtained from young healthy African Americans using commercially available isolation kits. Written informed consents were obtained from all participating donors. Protein marker expression of each fraction was analysed by Western blotting. Lipidomic analysis was performed using LC-MS operating in negative ion mode.

Results: Successful EVs, HDL and LDL/VLDL isolations were validated by confirming corresponding marker proteins (CD9; EVs, apoA-I; HDL, apoB; LDL/VLDL). As a result of lipidomic analysis, we identified 264 lipids in plasma EVs, HDL and LDL/VLDL fractions. We also found that EVs showed strikingly higher levels of lyso-glycerophospholipids than HDL and LDL/VLDL. Additionally, compared with EVs, higher sphiongolipid species levels were observed in LDL/VLDL, while polyunsaturated phosphatidylcholine were highly detected in HDL. Similar profiles were also observed in each fraction derived from human serum.

Summary/conclusion: Lipidomic profiling demonstrates that EVs has a unique lipid profile compared with lipoprotein particles, although the biological meaning of these differences should be further evaluated in future studies. Nevertheless, the method presented in this study can be useful for lipid biomarker screening for EVs as well as lipoprotein particles derived from both plasma and serum for human diseases.

Funding: Japan Agency for Medical Research and Development

LBT01.06

Enhancing extracellular vesicle isolation of human plasma verified by high resolution lipidomics

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Introduction: Extracellular vesicles (EVs) are secreted from many cell types and play important roles in intercellular communication. EVs carry a range of biomolecules that reflect the identity and molecular state

of their parental cell and are found in biological fluids. Omics studies have extensively focused on characterisation of the protein and nucleic acid cargo of EVs while lipids are less studied. EVs are increasingly being utilised in disease diagnosis as they are considered to carry valuable information about the disease state. Thus, novel disease biomarkers might be identified EV lipidomes.

Methods: EVs were enriched from 1ml normal human plasma samples using ultracentrifugation (UC), considered the gold standard approach for EV enrichment, and size exclusion chromatography (SEC) using qEV original columns (Izon, NZ). Lipids extracted according to Matyash et al. (2008) were loaded on a C30 Acclaim column (Thermo, AU) using a Vanquish liquid chromatography (LC) system and analysed using a Fusion orbitrap mass spectrometer (MS) using targeted and untargeted lipidomics approaches. LipidSearch software was used to annotate and quantify lipid species.

Results: More than 250 lipid species were identified and quantified in the plasma EVs following both enrichment methods. The two methods also generated highly similar lipid profiles, indicating that SEC may be a viable alternative to the cumbersome UC method. Interestingly, the SEC approach yielded less lysophosphatidylcholine (LPC) lipids, which may be related to a more homogenous vesicle population captured by SEC. Various literature reviews refer to glycerolipids, likely originating from co-isolating vesicles such as low-density lipoproteins, as contaminants in the EV fractions. We detected these lipids and propose that if they are differentially expressed in states of disease, they can be used as biomarkers independent of their origin.

Summary/conclusion: This study presents a workflow for comprehensive lipidomics of EVs using two isolation methods that are compatible with downstream state-of-the art LCMS, improving our ability to study the lipid components of EVs and identifying new disease biomarkers. As lipidome profiles were similar between the two isolation methods, large scale diagnostic assays should consider employing the SEC, which is by far the more efficient, scalable approach.

LBT01.07

Extracellular vesicle measurements with nanoparticle tracking analysis – An accuracy and repeatability comparison between NanoSight NS300 and ZetaView

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Introduction: The expanding field of extracellular vesicle (EV) research needs reproducible and accurate methods to characterize single EVs. Nanoparticle Tracking Analysis (NTA) is commonly used to determine EV concentration and diameter. As the EV field is lacking methods to easily confirm and validate NTA data, questioning the reliability of measurements remains highly important. In this regard, a comparison addressing measurement quality between different NTA devices such as Malvern's NanoSight NS300 or Particle Metrix' ZetaView has not yet been conducted.

Methods: To evaluate the accuracy and repeatability of size and concentration determinations of both devices, we employed comparative methods including transmission electron microscopy (TEM) and single particle interferometric reflectance imaging sensing (SP-IRIS) by ExoView. Multiple test measurements with nanospheres, liposomes and ultracentrifuged EVs from human serum and cell culture supernatant were performed. Additionally, serial dilutions and freeze-thaw cycle-dependent EV decrease were measured to determine the robustness of each system.

Results: Strikingly, NanoSight NS300 exhibited a 2.0–2.1-fold overestimation of polystyrene and silica nanosphere concentration. By measuring serial dilutions of EV samples, we demonstrated higher accuracy in concentration determination by ZetaView (% BIAS range: 2.7–8.5) in comparison to NanoSight NS300 (% BIAS range: 32.9–36.8). The concentration measurements by ZetaView were also more precise (% CV range: 0.0–4.7) than measurements by NanoSight NS300 (% CV range: 5.4–10.7). On the contrary, quantitative TEM imaging indicated more accurate EV sizing by NanoSight NS300 (% DTEM range: 79.5–134.3) compared to ZetaView (% DTEM range: 111.8–205.7), while being equally repeatable (NanoSight NS300% CV range: 0.8–6.7; ZetaView: 1.4–7.8). However, both devices failed to report a peak EV diameter below 60 nm compared to TEM and SP-IRIS.

Summary/conclusion: Taken together, NTA devices differ strongly in their hardware and software affecting measuring results. ZetaView provided a more accurate and repeatable depiction of EV concentration, whereas NanoSight NS300 supplied size measurements of higher resolution.

LBT01.09

Exodisc for fast and robust isolation of extracellular vesicles from whole-blood

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Introduction: The circulating nano-vesicles, known as extracellular vesicles, are abundant in most of the body fluids and play vital roles in regulation of various biological processes, including signalling in the tumour microenvironment. They possess significant potential for disease diagnosis and treatment monitoring, however, their use in clinical settings is limited due to lack of simple and robust isolation methods. To address this, earlier we have developed Exodisc for isolation and analysis of the EVs from urine. In this study, lab-on-a-disc for the isolation of EVs from whole blood, Exodisc-B, is demonstrated.
Methods: Exodisc-B comprises of blood separation and filtration chambers connected with individually addressable diaphragm valves for the automatic control of sequential transfer of liquid samples. The device consists of two nano-porous membrane filters with pore sizes of 600 nm (track-etched PC membrane) and 100 nm (AAO membrane). First, the plasma was separated and passed through two filters sequentially to concentrate the EVs on filter-II. Then the EVs were washed and transferred to a collection chamber for retrieval. The performance of the device in comparison to ultracentrifugation (UC) was evaluated by analysing yield, purity, RNA and protein content of the isolated EVs.
Results: Compared with the UC technique, the Exodisc-B is capable of isolating at least an order of magnitude higher number of EVs with about 30-fold higher mRNA count within 40 min. Sandwich ELISA of EV-specific membrane proteins – CD9-CD81 – confirmed that it can isolate EVs with a capture efficiency >75%. The device also facilitates temporal monitoring of tumour progression within live mouse xenograft models over a period of 13 weeks while using minimal volumes of weekly collected blood samples. Further, in ELISA analyses of multiple cancer-related proteins extracted from EVs isolated from human plasma, 43 patients were differentiated from 30 healthy donors.
Summary/conclusion: We have demonstrated the performance of Exodisc-B for label-free and automatic isolation of EVs from whole-blood. The device offers a simple, fast and efficient means of intact EV isolation in a reproducible manner, from small sample volumes measuring as small as 30 μL of whole-blood.
Funding: This work was supported by grants A121994 and IBS-R020-D1 funded by the Korean Government.

LBT01.10

Optimization and characterization of low vacuum filtration procedure – novel method for the isolation of extracellular vesicles

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Introduction: Despite recent developments in the field of extracellular vesicles (EVs) isolation methods, the process remains challenging, mainly due to the low isolation yield, co-precipitation of proteins, changes in biophysical properties of EVs and time consuming procedures. Answering these problems, we created and validated new EVs isolation method – Low Vacuum Filtration (LVF) and compared it with two most commonly applied procedures – differential centrifugation (DC) and ultracentrifugation (UC).
Methods: The main element of the isolation system is dialysis membrane (MWCO = 1,000 kDa) combined with the low vacuum pump, assuring the high yield of isolation and short procedure time. EVs isolated from endothelial cells culture media have been characterized by (a) transmission electron microscopy (TEM) (b) nanoparticle tracking analysis (NTA), (c) western blot and (d) Fourier-Transform Infrared Spectroscopy (FTIR).
Results: TEM measurement visualized EVs with size of (a) LVF: 201 ± 136 nm, (b) DC: 256 ± 140 nm and (c) UC: 78 ± 25 nm. For LVF and DC EVs size was confirmed by NTA, for UC estimated size was higher (224 ± 112 nm). NTA showed substantial increase in EVs concentration, compared to the initial sample: (a) LVF: 22 fold, (b) DC: 13 fold, (c) UC: 35 fold. Western blot analysis confirmed the presence of exosome's (hsp70) and ectosome's (Arf6) markers in (a) LVF – CHsp70 = 0.48 ± 0.14 AU and CArf6 = 0.05 ± 0.02 AU, (b) DC – CHsp70 = 0.04 ± 0.01 AU and CArf6 = 0.07 ± 0.02 AU) and (c) UC (CHsp70 = 0.23 ± 0.12 AU and CArf6 = 0.07 ± 0.04 AU). We observed correlation between ATR-FTIR

spectra quality (amid I:lipids ratio) and the EVs and proteins concentration.

Summary/conclusion: LVF method is an easy and fast EVs isolation method which allows for isolation of both ectosomes and exosomes from high volume sources and could be an efficient alternative for commonly applied methods.

Funding: The authors acknowledge financial support from National Science Centre Poland [grant no. 2017/25/N/ST5/00831].

LBT01.11

Heterogeneity of erythrocytes derived microvesicles: their size and concentration

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Introduction: Extracellular Vesicles (EV) are heterogeneous populations of vesicles with different compositions, physiochemical properties and sizes. Random sampling is convenient when the population members are similar to one another on variables, ensuring a high degree of representativeness because it can critically construct generalizability of results. These key concepts can be misconstrued and blended, concealing the general drawbacks of validity. Here, erythrocytes MV (eMV) samples were derived from the same blood donor and are not to be presented as representative data of the general type of MV population, but to compare their heterogeneity (induced and non-induced).

Methods: Blood cells were purified and isolated utilising the Ficoll-Paque PLUS method. Erythrocytes concentrate pellets were used in 3 different ways (in triplicates): 1. eMVc- erythrocyte pellet with 10 mL 200 nm filtered PBS pH 7.4; 2. eMV CaCl2- As in 1 plus 20 µL of 2 mM of CaCl2 and; 3. eMV NHS- as in 2 plus 1 mL 20 nm filtered normal human serum (NHS). Samples were incubated for 45mins at 37°C and eMV were isolated by ultracentrifugation and suspended in 200 nm filtered PBS then analysed using the Guava flow cytometry (FC) EasyCyte HT system and qNano instrument.

Results: FC analyses showed similar characteristics in sizing and FC position (>800nm), the majority of samples laying within the MV gate created (size beads used), but eMV CaCl2 (3.4x10 6/mL) and eMV NHS (2.9x 10 7/mL) showed a significant increase in their amount of release (*P* value/mL). qNano analyses showed an increase in concentration distribution for induced samples (100nm to 800nm). eMVc peaks were concentrated between 170 nm to 220 nm (ranging 100 nm to 370 nm). eMV CaCl2 showed a greater concentration between 150 nm to 250 nm (ranging 100 nm to 600 nm) and eMV NHS was between 120 nm to 350 nm (ranging 100 nm to 750 nm).

Summary/conclusion: Here, the FC size beads proved to be useful not only for the measurement of MV sizes, but also for the concentration as MV samples fitted with the created gate. In relation to MV concentration distribution, the qNano proved to be a helpful apparatus by accurately distributing the concentration of MV according to their size spectrum. A combination of the different approaches may be able to provide useful information on the amount of MV released from inducements.

LBT01.12

Pentapartite fractionation of particles in oral fluids by differential centrifugation

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Introduction: Novel diagnostic methods are being developed for various oral maladies by using extracellular vesicles (EVs) contained in oral fluids because EVs carry condensed diagnostic information. However, our knowledge on the comprehensiveness of oral EVs is still very limited. In particular, cross-contamination of desquamated epithelial cells in oral fluids with EV fractions is our current interest because this contamination could interfere with the diagnostic information. To understand the possible interference of desquamated epithelial cells in oral EVs, we fractionated human oral fluids into 5 fractions by differential centrifugation and analysed the protein markers and nucleic acids in the fractions.

Methods: We obtained oral fluids from three healthy volunteers with informed consent. Each sample was separated into 5 fractions (0.3K, 2K, 10K, 160K and supernatant) by differential centrifugation. The numbers and the sizes of the particles in the fractions were analysed by nanoparticle tracking analysis (NTA). The expression levels of the protein markers were estimated by western blotting (WB). The amounts of mitochondrial and bacterial DNAs were quantified by PCR-based methods targeting the ND1 gene and rRNA gene, respectively. The numbers of cells were estimated by Trypan blue and Papanicolaou staining.

Results: Trypan blue staining showed that the 0.3K and 2K fractions contained 1.35×10^5 and 2.22×10^2 cells/mL of nucleated cells, respectively, while no intact cell was observed in the 10K and 160K fractions by Papanicolaou staining. NTA showed that the average diameters of the particles in the 10K, 160K, and the supernatant were 206.1 ± 17.0 nm, 122.1 ± 9.2 nm and 139.4 ± 29.4 nm, respectively. WB analyses showed that CD81, CD9, Alix, and Aquaporin 5 were mostly enriched in the 160K fraction, whereas HSP70, Ago2, and ATP5A were the most abundant in the 0.3K fraction. Mitochondrial DNA was abundant in the 0.3K fraction, and bacterial ribosomal DNAs were present in the 0.3K and 2K fractions.
Summary/conclusion: The WB suggested that HSP70, Ago2, and ATP5A can be used as markers of whole cells (mostly desquamated cells). Because the expression levels of these markers in 10K and 160K were very limited, we concluded that cross-contamination of desquamated epithelial cell-derived particles in 10K and 160K would be very less, if any.

LBT01.13

Heat shock protein-accessorized exosomes: presence in states of danger, disease, and disruption

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Introduction: Heat shock proteins (HSPs) function as chaperones under both normal and pathologic conditions. As chaperones they assist in protein folding, in holding protein complexes for current or future activation, and in the degradation of senescent proteins for recycling of components and displaying for immune surveillance. During stressful situations, HSP quantities and/or activities are increased as cells and tissues seek protection from insults. On occasion, these insults can result in the cell surface display of HSPs, which can then lead to the surface display of HSPs on exosomes, membrane-enclosed vesicles released extracellularly after passage through the endosomal system. HSPs present on the cell surface or in the extracellular space are regarded as "danger signals" in an ancient biologic paradigm. HSP-accessorized exosomes may act as "danger boli", carrying not only the HSPs, but hundreds of components of the stressed parental cell, capable of prompting immune responses, or possibly immune suppression, depending on the status of the recipient cell.
Methods: Exosomes from the plasma of patients suffering from neurological maladies (glioblastoma grade IV, traumatic brain injury, multiple sclerosis) are precipitated by peptides designed to bind HSPs and analysed by metabolomics.
Results: The metabolome of exosomes purified by HSP peptides from plasma of patients with various neurological disorders is distinct from that of blood exosomes from healthy donors (>80 distinct compounds in GBM exosomes, and TBI exosomes; >30 compounds in MS exosomes; all are unique to those groups). There are also numerous lipid and metabolic pathways linked to those compounds.
Summary/conclusion: Such HSP-accessorized exosomes thus possess metabolites with possible ties to the different CNS pathologies that may represent disease-specific biomarkers in a "liquid biopsy" setting.

LBT02: Late Breaking- EV Biomarkers
Chairs: Maja Mustapic; Dakota Gustafson
Location: Level 3, Hall A

15:30–16:30

LBT02.01

Cancer stem cell-derived exosomes potential for early detection in pancreatic cancer

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Introduction: Pancreatic cancer (PaCa) is the most deadly malignancy, due to late diagnosis and early metastatic spread, which prohibits surgery. it is urgently for reliable, early detection. Research shows that tumour-derived exosomes, which had been present in the blood in the early stage of tumour formation and before metastasis, is the vanguard forces of tumour formation and metastasis; Cancer stem cell-derived exosomes (CSC-Exos) has stronger migration ability, so the detection of blood CSC-Exos for early diagnosis and monitoring of progress for PaCa has great research potential and the value of application.

Methods: Protein markers were selected according to expression in exosomes of PaCa cell line culture supernatants, but not healthy donors' serum- exosomes. According to these preselections, serum-exosomes were tested by flow cytometry for the pancreatic cancer stem cell marker Tspan8.

Results: The majority (95%) of patients with PaCa and patients with nonPa-malignancies reacted with anti-Tspan8. Serum-exosomes of healthy donors' and patients with nonmalignant diseases were not reactive. Recovery was tumour grading and staging independent including early stages.

Summary/conclusion: Thus, the evaluation of pancreatic CSC-derived exosomes awaits retrospective analyses of larger cohorts, as it should allow for a highly sensi- tive, minimally-invasive PaCa diagnostics.

Funding: Supported by the National Natural Science Foundation of China (No. 81702963)

LBT02.02=OWP3.01

Using plasma to identify neural biomarker for antidepressant response in a treatment resistant cohort

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Introduction: Small extracellular vesicles (SEV) have emerged as candidate biomarkers in many complex diseases. An important characteristic of SEVs is their ability to bidirectionally cross the blood-brain barrier. This is particularly important in the context of major depressive disorder (MDD), where biomarkers are obtained from peripheral tissue and have been hard to relate to changes in brain functioning. 60% of MDD patients do not respond to their first antidepressant drug therapy (ADT) and treatment options are entirely at the discretion of the physician. Findings that can predict ADT response as well as provide insight into central mechanistic changes could revolutionize MDD treatment. The aim of this study is to profile exosomal microRNA (miRNA) in the context of ADT response in people with treatment-resistant depression. miRNA can act as biomarkers and might influence recipient cells to provide insight on disease-relevant mechanistic changes.

Methods: This pilot uses plasma from 10 controls and 10 patients with MDD (5 ADT responders (RES), and 5 non-responders (NRES)) from baseline (T0, before treatment). SEVs were isolated using a size exclusion column from Izon Science (Christchurch, New Zealand). Each isolation was divided into a "whole exosome" fraction and an immunoprecipitated "(NDE)" fraction using neural marker L1CAM. Quantitation and size determination was done using Tunable Resistive Pulse Sensing (TRPS) on the qNano gold. RNA was also extracted from SEVs from both fractions. The 4N-small RNA-Seq (Galas) protocol was used for library preparation.

Results: We found that the range of SEVs in the NDE fraction was smaller than the pool of all exosomes combined. Further SEVs from all depressed patients were significantly smaller than controls irrespective of the fractions. Our sequencing results showed an increase of miR-151a-3p and miR-3168 in NRES, and miR-22-3p in RES. These results were specific to the NDE fraction.
Summary/conclusion: We have identified three potential biomarkers for ADT response which are uniquely present in the neural-derived fraction of peripheral SEVs.
Funding: Canadian Institutes of Health Research

LBT02.03=OWP1.08

Isolation of neuron-specific extracellular vesicles

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Introduction: Human biological fluids contain extracellular vesicles (EVs) from different cell types. It would be incredibly useful to be able to isolate EVs that originated from specific cell types for diagnostic purposes as a way to gain molecular information (RNA, protein) from inaccessible cell types non-invasively.
Methods: We have developed a general framework for identifying EV surface markers that can be used for immuno-isolation of cell type specific EVs. As a proof of principle, we have applied this framework to the isolation of neuron-derived EVs from human cerebrospinal fluid or plasma. In addition to the computational analysis, we have developed an *in-vitro* system of human neurons differentiated from human induced pluripotent (iPS) cells. We performed mass spectrometry on EVs isolated from these neurons to identify neuron-specific proteins. We also used this system to develop a robust immune-isolation method for neuron EV markers.
Results: We have characterized the proteins present in neuron exosomes by mass spectrometry and then used computational analysis of published gene expression and proteomics data to come up with a list of candidate neuron-specific EV markers. After developing methods for immuno-isolation of neuron EVs with these markers, we applied our methods to human cerebrospinal fluid and plasma.
Summary/conclusion: We have developed a framework for the isolation of cell type specific EVs through the combination of an experimental *in vitro* system and computational analysis of gene expression and proteomics data. We have applied this framework to the isolation of neuron-specific EVs in human biological fluids. We envision these methods being broadly applicable to the development of novel diagnostic biomarkers for a variety of diseases.

LBT02.04

Labelling and tracking extracellular vesicles using a RNA-targeting AIE fluorogen

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Introduction: Extracellular vesicles (EVs) are considered as crucial carriers in cell-to-cell communication, immune response, tumourigenesis and metastasis. To gain direct insights into EVs functions, it is necessary to observe their intracellular localizations and biodistribution. Given the fact that EVs carry various RNA species, fluorescence labelling of RNA in EVs is one of the most high-profile strategies. However, ideal probes are still lacking.
Methods: In this work, we report that a commercial cell-permeant dye HSP may serve as a simple and facile probe for staining RNA within EVs. The good performance of HSP allows EVs to be analysed and imaged by nano-flowcytometry and structured illumination microscopy (SIM), respectively. Additionally, for the first time we uncover that HSP exhibits typical AIE (aggregation-induced emission) property. The labelling procedure can thus be performed in a wash-free manner due to the low fluorescent background of HSP in water before binding to RNA, which greatly avoid EVs losing during the experiment.
Results: HSP shows advantages over traditional SytoRNASelect in labelling EVs RNA in terms of its superior brightness, high specificity and excellent photostability.
Summary/conclusion: HSP may serve as a new probe for EVs labelling and shows great potential in studying behaviours and bio-distributions of EVs in a wide range of research fields.

LBT02.05

The identification of extracellular vesicles proteins in glioblastoma diagnosis

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Introduction: Glioblastoma multiforme (GBM) is a highly malignant type of brain tumour in humans. GBM cells reproduce quickly and the median survival time for patients is about 1 ~ 2 years. Current diagnostics and treatments for GBM are limited. Recently, many studies used proteomic analyses of GBM extracellular vesicles (EVs) or secretomes have been helpful in identifying biomarkers and potential treatment strategies for GBM.
Methods: Herein, our study used mass spectrometry (MS) to analysis the EV proteins from GBM cell lines – U87 and A172, and normal human astrocyte – SVG-p12 cultures. IPA analysis identified several proteins from GBM cell lines EVs are significantly different from the normal astrocytes cultures. EVs from 30 patients plasma with different grades of glioma were isolated and analysed to conform the findings from IPA analysis
Results: We identified several signalling pathways have been changed in different GBM cultures. Further validation with 30 different grade of glioma patients, we identified three proteins – chaperonin containing TCP1 subunit 8 (CCT8), Glypican (GPC1) and Periostin (POSTN) which levels in plasma EVs are associated to GBM but not plasma which also have been reported associated to GBM progression. Database analysis also found the EVs level of CCT8, GPC1 and POSTN in different grade of glioma can represent the RNA level in tumour from microarray. Additionally, we also found some specific signalling pathways changes in different GBM lines such as transforming growth factor beta induced (TGFB1) in U87 EVs and prosaposin (PSAP) in A172 EVs. The elevation of different molecules in EVs provides specific characters to individual GBM.
Summary/conclusion: We found EV contents – CCT8, GPC1 and POSTN were associated in GBM which could be used for clinical diagnosis; also some different GBM EV proteins – TGB1 and prosaposin could be used in characterization and targeting therapy of GBM in the further.
Funding: Ministry of Science Technology – MOST 105-2628-B-038-005-MY3

LBT02.06

Universal reference transcripts for miRNA normalization – a meta-analysis on human blood extracellular vesicle RNA sequencing data sets

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Introduction: Due to their importance in intercellular communication, extracellular vesicles (EV) have emerged as important sources of biomarkers for pro- and diagnostic purposes. With the advent of RNA-seq as the tool of choice for unbiased biomarker screening, a major focus has been laid on miRNAs, important regulators of post-transcriptional gene expression. Feasibility of RNA biomarkers presently still relies on validation and analysis by RT-qPCR which in turn is depending on stably expressed reference transcripts for normalization. To assess whether a set of universal reference miRNA transcripts for normalization exists, a meta-analysis on blood derived EV samples was conducted.
Methods: From eight different research studies, we analysed small RNA-seq reads of 531 EV samples that were isolated from various pathological conditions or healthy controls and enriched by standardized methods (SEC, UC or precipitation). To account for the variety of commonly utilized RNA-seq analysis methods, a standardized big-data analysis pipeline was established, that combined robust filtering by six different normalization methods and three algorithms to detect suitable reference transcripts. Sets of stably expressed transcripts were finally compared across different studies, isolation methods and data analysis combinations.
Results: Results of our pipeline showed substantial overlap for miRNAs ranked by stability for different normalizations and algorithms over all samples albeit compromised by high variances in general. Contrarily reference miRNAs determined within a single research study showed much higher stability values and were consistent over multiple analysis combinations.
Summary/conclusion: Although first results suggest the possibility that blood EVs contain a common set of miRNAs that may be used as universal reference transcripts, different EV isolation methods, pathophysiological conditions and sequencing methodology have a major influence on expression profiles. With the availability of additional small RNA-seq data sets in the future, robustness and validity of miRNA references will continue to grow, but success will ultimately be depending on further standardization of EV isolation and data analysis. Based on the current data, we recommend analysing reference transcripts in each study individually.

LBT02.07

Small extracellular vesicle content in porcine blood plasma, cerebrospinal fluid and seminal plasma for proteomic analyses in biomarker discovery

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Introduction: Extracellular vesicles (EVs) released to body fluids carry molecules of the source cells and are subjects of intensive research of protein and nucleic acid biomarkers of diseases. Pig represents a valuable experimental biomedical model to study human diseases due to close anatomic and physiologic similarity to human. The aim of this work was to compare suitability of porcine blood plasma, cerebrospinal fluid and seminal plasma for EV isolation for proteomic analyses and optimize sample preparation for mass spectrometry.
Methods: EVs were isolated from porcine body fluids by differential centrifugation and ultracentrifugation and characterized by transmission electron microscopy, flow cytometry and western blotting. Three different lysis buffers (RIPA, Triton X100 and SDS) were compared in efficacy to extract EV proteins in combination with filter-aided sample preparation (FASP) for LC-MS/MS analysis (triple TOF).
Results: Seminal plasma yielded largest amount of EVs, followed by blood plasma. In cerebrospinal fluid, the EV content was very low. Proteomic analysis of seminal plasma-derived EVs enabled identification of approximately 1200 proteins, including 76 of the top 100 mainly identified proteins in EVs (Exocarta). Approximately 550 proteins were quantified by SWATH-MS. In contrast, only 200 proteins were identified in the crude seminal plasma used for EV isolation.
Summary/conclusion: We have optimized techniques for the EV enrichment from porcine body fluids and for characterization of their protein content by mass spectrometry. Such techniques may be applied to biomarker discovery in porcine model of diseases as well as adopted to other species, including human.
Funding: This study was supported by Czech Science Foundation (reg. No. 19-01747S), Operational Programme Research, Development and Education (reg. No. CZ.02.1.01/0.0/0.0/16_019/0000785), and National Sustainability Programme I. of the Czech Ministry of Education, Youth and Sports (reg. No. LO1609).

LBT02.08

Quantitative proteomic profiling of tissue-exudative EVs identified a novel diagnostic antigen for early detection of colorectal cancer

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Introduction: Development of biomarkers for early detection of colorectal cancer (CRC) is demanded as the number of CRC patients is increasing. Recent studies exhibit that extracellular vesicles (EVs) are expected as biomarker carriers in any body fluids. To explore CRC-specific antigens, we isolated EVs from viable CRC or adjacent normal tissues (n = 17), followed by global quantitative proteome analysis.
Methods: Tissue-exudative EVs (Te-EVs) were purified from serum-free media of freshly resected CRC and adjacent normal tissues, using the sequential ultracentrifugation method (n = 17). Purified Te-EVs were analysed by Orbitrap Fusion Lumos LC/MS system (Thermo Scientific). Protein identification, label-free quantification, and statistical analysis were performed on MaxQuant and Proteome Discoverer softwares. A statistically valid biomarker candidate protein (TMAM) was further evaluated by plasma exosome sandwich ELISA (n = 357). Additional clinical and functional assessments were also performed including IHC staining and cell growth assays.
Results: Among 6,149 identified Te-EV proteins, 393 proteins were significantly overexpressed (p < .05 and fold change > 4.0) in EVs from CRC tissues compared to those from normal mucosa. We especially focused on transmembrane protein TMAM (p = 3.62 E-5, fold change = 7.0) which was known to be a key regulator of cell growth and also overexpressed in CRC cells. Exosome sandwich ELISA confirmed significant elevation of TMAM level in plasma EVs even in stage-I CRC patients (n = 72) compared to healthy donors (n = 72, p = .040). IHC staining analysis also showed that TMAM was specifically overexpressed in CRC tissues. Interestingly, TMAM-overexpressed EVs decoyed its inhibitory ligand away from cancer cells, resulting in their outgrowth.
Summary/conclusion: These results indicate that TMAM on EVs should have great potential as a novel target for CRC diagnosis and therapy.

LBT02.09

Single-molecule co-Immunoprecipitation reveals functional inheritance of epidermal growth factor receptors in extracellular vesicles

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Introduction: Cancer cells actively release extracellular vesicles (EVs) as important carriers of cellular information to tumour microenvironments. Although the composition and quantity of the proteins contained in EVs are characterized, it remains unknown how these proteins in EVs are related to those in the original cells at the functional level. Ultimately, the question should be resolved to ensure the use of EVs in diagnosing the status of cancer patients by liquid biopsy.

Methods: Using the recently developed single-molecule immunolabelling and co-immunoprecipitation schemes, the quantity and PPI strengths of EGFRs derived from EVs and the original lung adenocarcinoma cells are determined.

Results: It is found that the microvesicles exhibit higher correlations with the original cells than the exosomes in terms of the EGFR levels and their PPI patterns. In spite of these detailed differences between the microvesicles and exosomes, the EGFR PPI strengths measured for EVs generally show a tight correlation with those determined for the original cells.

Summary/conclusion: With epidermal growth factor receptor (EGFR) in lung adenocarcinoma cells as a model oncoprotein, it is studied how distinct types of EVs, microvesicles and exosomes, represent their original cells at the protein and protein–protein interaction (PPI) level. The results suggest that EGFRs contained in EVs closely reflect the cellular EGFR in terms of their downstream signalling capacity. Moreover, it gives a possibility that EGFRs derived from different types of EVs may work as a biomarker for the intensity of the EGFR signalling pathway in the parental cancer tissue.

LBT03: Late Breaking- EVs and Stem Cells
Chairs: Sicheng Wen; Hiroaki Tateno
Location: Level 3, Hall A

15:30–16:30

LBT03.01

Regenerative potential of extracellular vesicles-derived from mesenchymal stem cells on epithelial wound healing

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Introduction: Wound healing is a complex process involving cell death, migration, proliferation, differentiation, inflammation, and extracellular matrix remodelling. A key role in this context is played by resident stem cells. Mesenchymal stem cells (MSCs) favour wound healing via extracellular vesicles (EVs), which transfer transcription modulators and nucleic acid, including mRNA and micro-RNA.

Methods: We found that MSC-derived EVs favour epithelial wound healing *in vitro*, and that EVs regulate the EGFR/PI3K/Akt/mTOR pathway, a key player in keratinocyte stem cells biology, glucose homeostasis and aging. Moreover we have characterized the mRNA and miRNA content of MSC-derived EVs and our analysis revealed several miRNA potentially involved in wound healing. To identify potential miR candidates, we clustered miRNAs expressed by EVs into families, according to their seed sequence and scanned the 3′-UTR of keratinocyte expressed genes for perfect seed-match occurrences. To account for potential cooperative action of different miRNAs, we will restrict our research to those genes targeted by at least 2 expressed miRNA families.

Results: We selected several miRNAs which target wound healing cellular pathways and carried by MSC-EVs (miR-let-7a-5p, miR-10a-5p, miR-10b-5p, miR-21-5p, miR-22-3p, miR-100-5p, miR-143-3p, miR-146a, miR-191-5p, miR-181a-5p, miR-27b-3p). We have found that miRNA146a is a key activator of the Notch1/Akt pathway. Notably, Notch1 levels are elevated in limbal-corneal epithelial stem cells relative to their migratory cell progenies, and abnormally elevated miRNA146a levels are implicated in defective corneal wound healing in diabetes. Thus, miRNA146a may regulate the balance between LESC self-renewal versus migration/differentiation via Notch/Akt regulation.

Summary/conclusion: We have shown that transfection of MSC by siRNA anti miR-146a decrease the biologic effect of MSC-EVs on migratory capacity of epithelial cells. It could be a direct effect of the absence of miR-146a in MSC-EVs or consequence of the miR-146a signalling pathway disruption.

Funding: CAMG_PRIN_2015_16_01

LBT03.02

Intravenous administration of xenogenic adipose-derived mesenchymal stem cells (ADMSC) and ADMSC-derived exosomes markedly reduced brain infarct volume and preserved neurological function in rat after acute ischemic stroke

Shun-Cheng Wu[a], Pei-Lin Shao[b] and Hon-Kan Yip[c]

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Introduction: We tested the hypothesis that combined xenogenic (from mini-pig) adipose-derived mesenchymal stem cell (ADMSC) and ADMSC-derived exosome therapy could reduce brain-infarct zone (BIZ) and enhance neurological recovery in rat after acute ischemic stroke (AIS) induced by 50-min left middle cerebral artery occlusion.

Methods: Adult-male Sprague-Dawley rats (n = 60) were divided equally into group 1 (sham-control), group 2 (AIS), group 3 [AIS-ADMSC (1.2 × 106 cells)], group 4 [AIS-exosome (100 μg)], and group 5 (AIS-exosome-ADMSC). All therapies were provided intravenously at 3h after AIS procedure.

Results: BIZ determined by histopathology (by day-60) and brain MRI (by day-28) were highest in group 2, lowest in group 1, higher in groups 3 and 4 than in group 5, but they showed no difference between groups 3 and 4 (all $p < .0001$). By day-28, sensorimotor functional results exhibited an opposite pattern to BIZ among the five groups ($p < .005$). Protein expressions of inflammatory (inducible nitric oxide synthase/tumour necrosis factor-α/nuclear factor-κB/interleukin-1β/matrix metalloproteinase-9/plasminogen activator inhibitor-1/RANTES), oxidative-stress (NOX-1/NOX-2/oxidized protein), apoptotic (caspase-3/Poly-

ADP-ribose polymerase), and fibrotic (Smad3/transforming growth factor-β) biomarkers, and cellular expressions of brain-damaged (γ-H2AX+/XRCC1-CD90+/p53BP1-CD90+), inflammatory (CD11+/CD68+/glial fibrillary acid protein+) and brain-oedema (aquaporin-4+) markers showed a similar pattern of BIZ among the groups (all $n < 0.0001$).

Summary/conclusion: In conclusion, xenogenic ADMSC/ADMSC-derived exosome therapy was safe and offered the additional benefit of reducing BIZ and improving neurological function in rat AIS.

LBT03.03

Changes in amino acid concentration of umbilical cord mesenchymal stem cell culture medium

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Introduction: Mesenchymal Stem Cell is a multipotent cell that works in two ways, replacing the injured cells and releasing active biomolecules that work as a paracrine signal for cell migration and proliferation. Recent discoveries suggest that potential cytokine, growth factors, and many different soluble factors are released by MSCs during the culturing process into its environment. In this study, we aim to analyse that changes of amino acid concentration from the fresh complete growth medium and post-culture medium from umbilical cord mesenchymal stem cell (UC-MSC) cultured.

Methods: UC-MSC was cultured with the seeding density of 5000 cells/cm^2 in tissue culture plasticware. When the cells, reached 70–80% confluency, the culture medium was collected and centrifuged to remove the unwanted debris. Collected medium was stored in −80°C until the amino acid concentration was analysed using Mass Spectrophotometry.

Results: The fresh and post-culture media contains both essential and non-essential amino acid. The post-culture culture media contains higher amino acid compared to the fresh medium. In this study, there is an increasing concentration of glycine, l-arginine, l-phenylalanine, l-histidine, l-leucine, l-lysine, l-serine, l-threonine, l-tyrosine and l-valine concentration. The concentration of L-glutamine from post-cultures is decreasing compared to fresh medium while the concentration of L-glutamic acid (+959 mg/ml) is increasing. This due to the regulation of glutamate synthase which changes the L-glutamine into L-glutamate (L-glutamic acid). The methionine and cysteine cycle also show by comparing both media, where methionine and cysteine are decreasing to produce cystine.

Summary/conclusion: Glutamate-Glutamine cycle and Methionine-Cysteine cycles were shown in the metabolism of UC-MSC proliferation during cell culture. This cause changes in amino acid concentration between fresh and post-culture media.

Funding: All the research were funded by PT. Prodia StemCell Indonesia

LBT03.04

Combination of adipose-derived mesenchymal stem cells (ADMSC) and ADMSC-derived exosomes for protecting kidney from acute ischemia-reperfusion injury

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Introduction: In this study, we tested the hypothesis that a combined adipose-derived mesenchymal stem cell (ADMSC) and ADMSC-derived exosome therapy protected rat kidney from acute ischemia-reperfusion (IR) injury (i.e., ligation of both renal arteries for 1h and reperfusion for 72h prior to euthanization).

Methods: Adult-male SD rats (n = 40) were equally categorized into group 1 (sham control), group 2 (IR), group 3 [IR+exosome (100 μg)], group 4 [IR+ADMSC (1.2 × 10(6) cells)] and group 5 (IR-exosome-ADMSC). All therapies were performed at 3 h after IR procedure from venous administration.

Results: By 72h, the creatinine level and kidney injury score were the lowest in group 1 and the highest in group 2, significantly higher in group 3 than in groups 4 and 5, and significantly higher in group 4 than in group 5 (all $P < .0001$). The protein expression of inflammatory (TNF-α/NF-κB/IL-1β/MIF/PAI-1/Cox-2), oxidative-stress (NOX-1/NOX-2/oxidized protein), apoptotic (Bax/caspase-3/PARP) and fibrotic (Smad3/TGF-β) biomarkers showed an identical pattern, whereas the anti-apoptotic (Smad1/5, BMP-2) and angiogenesis (CD31/vWF/angiopoietin) biomarkers and mitochondrial cytochrome-C showed an opposite pattern of creatinine level among the five groups (all $P < .001$). The microscopic findings of glomerular-damage (WT-1), renal tubular-damage (KIM-1), DNA-damage (γ-H2AX), inflammation (MPO/MIF/CD68) exhibited an identical pattern, whereas the podocyte components (podocin/p-cadherin/

synaptopodin) displayed a reversed pattern of creatinine level (all $P < .0001$).

Summary/conclusion: Combined exosome-ADMSC therapy was superior to either one for protecting kidney from acute IR injury.

LBT03.05

Adipose-derived mesenchymal stem cell-derived exosomes alleviate overwhelming systemic inflammatory reaction and organ damage and improve outcome in rat sepsis syndrome

Pei-Lin Shao[a], Shun-Cheng Wu[b] and Hon-Kan Yip[c]

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Introduction: This study tested the hypothesis that healthy adipose-derived mesenchymal stem cell (ADMSC)-derived exosomes ($HMSC^{EXO}$) and apoptotic (A) (induced by 12 h hypoxia/12 h starvation)-ADMSC-derived exosomes ($AMSC^{EXO}$) were comparably effective at alleviating sepsis syndrome [SS; induced by cecal-ligation and puncture (CLP)]-induced systemic inflammation and reduced organ damage and unfavourable outcomes in rats.

Methods: SD rats were divided into sham control (SC), SS only, SS + HMSCEXO (100 μg intravenous administration 3 h after CLP), and AMSCEXO.

Results: By day 5 after CLP procedure, the mortality rate was significantly higher in SS than in SC and HMSCEXO (all $P < .01$), but it showed no significant different between SC and HMSCEXO, between AMSCEXO and HMSCEXO or between SS and AMSCEXO ($P > .05$). The levels of inflammatory mediators in circulation (CD11b/c/Ly6G/MIF), bronchioalveolar lavage (CD11b/c/Ly6G) and abdominal ascites (CD11b/c/CD14/Ly6G/MIF) were highest in SS, lowest in SC and significantly higher in AMSCEXO than in HMSCEXO (all $P < .001$). The circulating/splenic levels of immune cells (CD34+/CD4+/CD3+/CD8+) were expressed in an identical pattern whereas the T-reg+ cells exhibited an opposite pattern of inflammation among the groups (all $P < .001$). The protein expressions of inflammation (MMP-9/MIF/TNF-α/NF-κB/IL-1β) and oxidative stress (NOX-1/NOX-2/oxidized protein), and cellular expressions (CD14+/CD68+) in lung/kidney parenchyma exhibited an identical pattern of inflammatory mediators (all $P < .001$). The kidney/lung injury scores displayed an identical pattern of inflammatory mediators among the groups (all $P < .001$).

Summary/conclusion: In conclusion, HMSCEXO might be superior to AMSCEXO for improving survival and suppressing the inflammatory reactions in rats after SS.

LBT03.06

Bile acids hybrid extracellular vesicles derived from mesenchymal stem cells for cartilage tissue regeneration

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Introduction: Tauroursodeoxycholic acids (TUDCA) has been known as an amphiphilic therapeutic drug for a variety of diseases such as cholestasis, amyotrophic lateral sclerosis, type 1 diabetes and so on. Recently, we reported TUDCA has a role in bone and cartilage regeneration through leading to osteogenic or chondrogenic differentiation of mesenchymal stem cells (MSCs). In addition, TUDCA is also able to form a nano-sized micelle, penetrate and incorporate into the membrane of cells depending on the concentration, therefore, suggesting that TUDCA would be a useful drug to modify cell membrane and extracellular vesicles (EVs).

Methods: In this study, we investigated whether the EVs derived from the amphiphilic bile acids-treated cells could produce hybrid EVs composed with cell membrane and bile acid and also they include mRNA, micro RNA and proteins at the core of EVs. To aim this, we isolated EVs from TUDCA-treated mesenchymal stem cells (MSCs) and identified their characteristics. In addition, the regenerative effect of EVs was also evaluated using degenerated chodnrocytes (DC) derived from the knee cartilage of osteoarthritis (OA) patients.

Results: It was found that TUDCA-hybrid EVs (TUDCA-EVs) was successfully isolated and the total amount of EVs was increased with the treatment of TUDCA. We also tried to characterize the size and zeta-potential of TUDCA-EVs as well as the expression of genes which are contained in TUDCA-EVs compared to control EVs. Next, TUDCA-EVs were treated to DC. TUDCA-EVs treatment on degenerated chondrocyte (DC) increased IL-6 expression associated with the differentiation of M2 macrophage and anti-inflammatory signalling. In addition, TUDCA-EVs increased COL2 expression while they decreased RUNX2 and MMP13, as a hypertrophic marker gene, expression in DC.

Summary/conclusion: These indicated that TUDCA-EVs have the potential to restore the chondrogenic properties of degenerated chondrocytes. Therefore, it is concluded that TUDCA-EVs would be a useful systemic drug for OA therapy. In ongoing *in vivo* study, we are confirming whether TUDCA-EVs indeed have an influence on cartilage tissue regeneration and alleviate OA symptoms.

LBT03.07

Role of stem cell-derived extracellular vesicles and their enriched microRNAs during alcoholic liver injury

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Introduction: Senescence of activated stellate cells limits hepatic fibrogenesis. Stem cell-derived extracellular vesicles (EVs) and their related microRNAs mediate genetic changes that promote recovery of liver disorders. The present study was aimed to characterize the functional role of liver stem cell-derived EVs and specific miRNAs in the regulation of hepatic stellate cell senescence during alcohol induced liver injury.

Methods: microRNA expression was assessed using microarray and real-time PCR assays in isolated EVs from human mesenchymal stem cells (MSCs) and liver stem cells (LSCs). HSCs were also isolated from lin28 knockout mice with or without ethanol feeding for 5 weeks by laser capture microdissection (LCM) and the senescence and fibrosis genes are evaluated by Western blot and real-time PCR analysis.

Results: We found that expression of several miRNAs were consistently up-regulated in both MSCs and LSC-derived EVs compared to normal hepatocyte-derived EV controls, including let-7 family members. Treatment of human HSCs with TGF-β/LPS (20 ng/ml) for 72 h induced a significant decrease of let-7a and let-7b in both activated and control states. Transfection of let-7a and let-7b precursors in human HSCs markedly induced the expression of cellular senescence markers p16 and CCl2, and blunted the enhanced expression of α-SMA, collagen a1, MMP-2 and MMP-9 (key genes involved in the activation of HHSCs) by TGF-β/LPS treatment. Treatment with MSC/LSC derived EVs (30 μg/ml, 72 h) phenocopied the senescence/anti-fibrosis effects of let-7 overexpression in activated HHSCs by TGF-β/LPS. A complementary mass spectrometry-based proteomics approach with luciferase reporter assay identified TLR4, the key LPS receptor, as putative let-7 cluster target. Furthermore, the expressions of senescent hepatic stellate markers and verified let-7 target genes, including α-SMA, collagen a1, p16, CCl2, TLR4, MMP-2, MMP-9 and TIMP-3, were significantly altered in liver specimens and LCM isolated HSCs from lin28 knockout mice with ethanol feeding relative to WT ALD mice controls.

Summary/conclusion: Our findings provide new insight into the function of specific miRNAs in stem cell-derived EVs regulating HSC senescence, and their therapeutic potentials in alcoholic liver injury and fibrosis.

LBT03.08

Interferon-gamma priming, but not hypoxia, modifies the miRNA landscape of human mesenchymal stromal cells (MSC) extracellular vesicles (EV)

Juliette Peltzer[a], Kyle Lund[b], Philippe Mauduit[c], Jean-jacques Lataillade[b] and Sebastien Banzet[d]

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Introduction: MSC-based cell therapy has received great interest in the past years, especially in regenerative medicine and tissue repair. The concept of priming consists in preconditioning the cells during the culture phase (often with cytokines or hypoxia) to improve their effects. The literature shows that MSC EVs can recapitulate a substantial part of the beneficial effects of the cells they originate from, and that miRNAs are key players in EVs action. Therefore, in the present work, our aim was to determine if IFN or hypoxia priming of MSC could modify their EVs miRNA content.

Methods: Human bone marrow MSC from five healthy donors were isolated and cultured at 20% of O2 in MEM-alpha/FBS medium until 60–70% confluence, then with (IFN) or without (CONT) interferon-gamma (25ng/ml, 48 h) or in hypoxia (3% O2 throughout the duration of the culture process). Then the cells were rinced with PBS and placed in serum free MEM for 48 h. The conditioned media was collected and EV were isolated by ultracentrifugation (100 000g for 1h10). Total RNA was isolated and reverse transcribed. Pools of CONT, IFN and HYP cDNA were prepared, miRNA profiling was performed using Exiqon miRnome PCR panel I and II. Then, selected miRNAs were measured on each sample.

Results: A set of 89 miRNAs was detected (quantification cycle < 35) in at least one of the pools of MSC EVs. They were measured on each individual sample. 41 miRNAs were measured in all samples; results were

normalized with 5 endogenous miRNAs. Hypoxia induced no significant modification of EVs miRNA content. IFN priming induced a significant increase in hsa-miR-106a-5p, 25-3p, 126-3p, 451a and 665. Their validated targets were determined with miRTarBase and the proteins were analysed with Panther classification system. Among the most cited pathways, we found p53, inflammation, Wnt signalling, Apoptosis signalling and Angiogenesis.

Summary/conclusion: MSC priming can modify the miRNA landscape of their EVs. IFN priming modifies MSCs EVs miRNA involved in biological pathways relevant to tissue repair. Functional analysis of these EVs with selected miRNAs inhibition is needed to evaluate the biological effects of such an approach.

Funding: This work has been funded by the french Direction Générale de l'Armement, Biomedef PDH-1-SMO-1–218

Industry Poster Session
Thursday 25 April 2019
Location: Level 3, Hall A

IP.01

Standardizing F-NTA measurements: evaluation of four-wavelengths nanoparticle tracking analysis with cell-line derived EVs

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Introduction: Nanoparticle tracking analysis (NTA) has emerged to a vital and fast characterization technology for exosomes, microvesicles or viruses. In combination with fluorescence detection (F-NTA), NTA enables the user to perform biomarkers detection on the single particle level, thus enhancing real EV concentration measurement. Classic NTA instruments are equipped with one laser, requiring phenotyping in sequence. Multi-fluorescence detection of four biomarkers in one sample by NTA is shown for the first time.
Methods: A four-laser NTA instrument (ZetaView PMX-420) equipped with excitation wavelengths of 405, 488, 520 and 640 nm and dedicated long-pass filters was evaluated. Concentration and particle size measurements were performed with fluorescent standard beads and proprietary labelled sub-micrometre sized vesicles. Phenotyping was performed on EVs from HCT116 cell line (HansaBioMed Life Sciences).
Results: The efficiencies of the individual laser channels were determined by fluorescently labelled vesicles. SOPs for conjugation of EVs were optimized regarding antibody to vesicle ratio and incubation time. Phenotyping by single and multi-wavelength NTA for wash and no-wash strategies were compared regarding background and efficiency.
Summary/conclusion: Standardization of SOPs is a key to improve repeatability for concentration measurements. Using four wavelengths, phenotyping of EVs was performed with four-fold reduction of sample amount in shorter time compared to sequential one laser measurements. NTA delivers total particle count, biomarker count and/or vesicle count on one sample including size distributions. Cross-validation with complementary techniques such as ELISA and FC/IFC becomes imperative.

IP.03

Double tangential flow filtration and size exclusion chromatography for scalable and reproducible EV isolation and size fractionation

Elina Aleksejeva[a], Julia Gavrilova[a], Maija Puhka[b], Karina A. Barreiro[c], Clemens Helmbrecht[d] and Paolo Guazzi[e]

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Introduction: The purification of Extracellular Vesicles (EVs) for industrial processes is still missing of reproducible, scalable and high throughput method, applicable to multiple sources of material (cell conditioned media, biofluids, plant extracts). HansaBioMed Life Sciences (HBM-LS) has developed a scalable EV purification process combining two tangential flow filtration steps followed by size exclusion chromatography. We set a standardized procedure which easily allows the isolation and the collection of big EVs (>200 nm), the fluid concentration and the removal of small molecules (< 500 kDa) with minimal loss of EVs, finally purified by SEC. The quality of vesicles has been assessed in terms of particle size distribution, morphology, concentration, phenotyping and storage stability.
Methods: EVs were isolated from cell conditioned media combining 2 TFF steps (HBM-TFF: HBM-TFF-MV) and SEC (maxiPURE-EVs HBM-LS). EV morphology and phenotype was analysed by NTA Zetaview (Particle Metrix), ExoTEST ELISA (HBM-LS), and electron microscopy.
Results: Analysing different purifications performed combining the double TFF and SEC we defined quality parameters for EVs in term of size distribution, concentration and maker expression, showing high reproducibility and EV stability under defined storage conditions.
Summary/conclusion: The combination of two TFF steps and SEC allows an efficient fractionation of different EV sizes and works as a scalable and reproducible method for EV production from large quantity of different fluids.

IP.04

Development of an automated, high-precision, standardizable extracellular vesicle isolation platform for clinical studies

Anoop Pal[a], Shayne Harrel[a], Robert Vogel[b] and Murray Broom[b]

[a]Izon Science US Ltd; [b]Izon Science Ltd

Introduction: Extracellular Vesicles (EVs) derived from biological fluids possess extensive heterogeneity with regards to size, number, membrane composition and cargo. Tremendous research interest exists towards development and use of EV fraction of bio-fluids as rich sources of diagnostic and prognostic biomarkers. High precision fractionation of the nanobiological content of biofluids can dramatically reduce background, increase purity and inform on the biology of the biomarkers and therapeutic biomolecules.
Methods: Size exclusion chromatography (SEC) is the most standardizable technique, already widely used for the purification of EVs from biofluids. Significant improvement to the use of SEC is possible through automation and precision. Here, we developed a range of SEC columns of various sizes, with 2 resin types, separating down to 35 nm or 70 nm. We also developed a low-cost prototype automatic fraction collector (AFC) that adds high precision, improves repeatability, speeds up workflow. RFID tags are proposed to ensure high quality of data capture and transfer. Moreover, Tunable Resistive Pulse Sensing technology was used for accurate, high-resolution particle analysis (size, size range, concentration, and electrophoretic mobility) and normalization.
Results: SEC columns provide a convenient, reproducible and highly effective means of eliminating >99% of non-vesicular protein from biological fluid samples, and separating exosomal and non-exosomal volumes for further downstream analysis. 35 nm pore sized SEC gel leads to increased resolution, higher yield and one fraction earlier elution of EVs from plasma compared to the 70 nm pore size. Use of AFC allowed precise mass-based measurements and tunability within 30 ul of volume exiting the column.
Most importantly, due to the additional functionality provided by AFC, the EV field needs to revisit the way fraction numbers, post-SEC are used. That will be replaced with a more logical framework, wherein the void volume is measured and disposed of, and precise volumes are used instead of the somewhat arbitrary fraction numbers.
Summary/conclusion: Thus, the qEV-AFC platform allows for QA, high-precision EV volume collection and minimizes samples processing related reproducibility issues for clinical studies.

IP.05

Faster, More Reproducible Exosomes Data – Hands Free!

Kohei Shiba, Pauline Carnell-Morris, Matthew McGann and Agnieszha Siupa

Malvern Panalytical

Introduction: In analytical data collection, the most common form of error is that generated by human error. From simple pipetting to manually adjusting optical settings on an instrument all these sources of error result in data sets that are less reproducible and increasingly difficult to interpret. The introduction of the NanoSight Sample Assistant for the NS300 brings about a new level of repeatability and reproducibility in analysis of Extracellular Vesicle (EV) samples. This work will examine the benefits of using the sample assistant for sample handling including time saving, and improved data quality.
Methods: The particle size distribution and concentration of exosome samples isolated from urine (20 x 1 mL) and SKOV3 cells (96 x 1 mL) was determined using the NanoSight NS300 system (Malvern Panalytical, UK) integrated with the NanoSight Sample Assistant (1mL). All samples were analysed under the same capture and process settings and the total time of analysis recorded. A series of experiments were also completed using SKOV3 samples, acquired manually on the NanoSight NS300 system to compare repeatability, reproducibility of data to that acquired by the sample assistant.
Results: Analysis of the data shows that data acquisition of 96 EV samples can be completed in approximately 15 h using the Sample Assistant, a 70% improvement compared to an estimated 50 h of manual acquisition. Setup time of the instrument however was approximately 30 min, reducing hands on instrument time by ~99%. An additional dataset of EV samples was measured as a dilution series, both manually and using the Sample Assistant. Data showed a measurable improvement in both repeatability of the concentration as well as linearity of the series.
Summary/conclusion: The new NanoSight sample assistant accessory for NS300 provides size and concentration data measurements of up to 96 samples in as little as 15 h, including under 30 min of set-up time. Data quality is typically improved by the elimination of user error and subjectivity. The Sample Assistant is compatible with many sample types, and generates

key exosome characterization data, whilst freeing up valuable scientist time to work on other tasks.

Funding: This project received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 646,002

IP.06

Identifying, characterizing and quantifying extracellular vesicles using multispectral imaging flow cytometry

Haley R. Pugsley, Sherree Friend, Bryan Davidson and Phil Morrissey

Amnis part of Merck KGaA

Introduction: Extracellular vesicles (EV) are a heterogeneous group of membrane derived structures that include exosomes, microvesicles and apoptotic bodies. Quantifying and characterizing EVs in a reproducible and reliable manner has been difficult due to their small size (down to 30 nm in diameter). Attempts to analyse EVs using traditional PMT based flow cytometers has been hampered by the limit of detection of such small particles, their low refractive index and the swarming effect. To overcome these limitations, we have employed multispectral imaging flow cytometry that has the advantage of high throughput flow cytometry with higher sensitivity to small particles due to the CCD based, time-delay-integration image capturing system. Several recent publications have reported using multispectral imaging flow cytometry to identify and characterize EVs; however, the collection settings and gating strategies used to identify and characterize EVs is not consistent between publications.

Methods: Here we demonstrate the optimal collection settings, parameters and gating strategy to identify, characterize and quantify a variety of EVs using multispectral imaging flow cytometry. EVs obtained from commercial sources are identified using a combination of CD markers, membrane stain and 405 nm SSC. In each case, the membrane stain and 405 nm SSC initially identify an EV and CD markers are used for characterization and immunophenotyping the EV.

Results: Data will be presented using the ImageStream multispectral imaging flow cytometer to identify, characterize and quantify a variety of EV samples. Strategies for optimal collection and analysis of the multispectral imaging flow cytometry EV data will also be discussed.

Summary/conclusion: Multispectral imaging flow cytometry is able to characterize and quantify EVs with very high sensitivity due to the CCD based time-delay-integration image capturing system.

IP.07

Microfluidic Resistive Pulse Sensing (MRPS) Measurements of EVs and EV Standards

Franklin Monzon[a], Jean-Luc Fraikin[b], Ngoc Do[a], Tom Maslanik[c], Erika Duggan[d] and John Nolan[d]

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Introduction: As science-based on EVs advances, it is important to be able to compare measurements of vesicles across different manufacturing sites and manufacturing methods. To isolate differences or drifts in EV formulations, it is necessary to have stable metrology so that these differences can be properly attributed to changes in the formulation and not the metrology. Establishing stable metrology in turn relies on the development of standards measured by multiple orthogonal methods. With this goal in mind, this paper discusses measurements of EVs and EV standards using Microfluidic Resistive Pulse Sensing (MRPS) and other measurement techniques.

Methods: The size distribution and concentration of EV standards and EVs derived from various sources were characterized by MRPS, Nanoparticle Tracking Analysis (NTA), cryo-Electron Microscopy (EM), and Vesicle Flow Cytometry (VFC). In some cases, EVs were destroyed by lysing agents and measurements were repeated to demonstrate this effect.

Results: MRPS measurements gave high resolution size and concentration information down to ~ 50 nm diameter for all samples. Because MRPS is an electrical technology, it did not suffer from sensitivity limitations related to the low index of refraction contrast between the nanoparticles (be they EVs or standards) and the surrounding liquid. MRPS could not distinguish particles based on type (in contrast to VFC), however it was more sensitive to the presence of non-EV nanoparticles in the samples. Concentration reproducibility was in the range of ± 20% and sizing reproducibility in the range of ± 5% independent of particle material.

Summary/conclusion: Quantifying the purity of an EV population is important. Techniques such as VFC do an excellent job in quantifying the EV population of interest but are not necessarily sensitive to contamination or the presence of non-target EVs. MRPS, on the other hand, gives high resolution information on all nanoparticles present in a mixture. From a process development standpoint, this information is critical to the improvement of a formulation. The orthogonal nature of MRPS measurements, compared to optical methods, is therefore an important part of the

development of robust EV standards, and the associated measurement protocols, that will be required for the successful wide deployment of EV-based diagnostics and therapeutics.

IP.08

Development of EV-targeting tools for novel liquid biopsy approaches in Lung Cancer

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Introduction: Mounting clinical evidence suggests that liquid biopsy may revolutionize the way cancer patients are currently managed. Within this context, our study aims to assess and reinforce unique and complementary advantages of EV/exosome-based approaches, through identification and quantitative detection of non-small cell lung cancer (NSCLC) EV biomarkers. Current technology and methods for exosome isolation from complex biological samples (i.e. plasma), have shown to be unreliable. There is a need to substantially improve them to enable multiparameter EV analysis. Therefore, in addition to EV-biomarker discovery, we are testing plasma processing and preanalytical tools, devices and optimized immunoaffinity protocols that tackle fundamental obstacles, such as complex matrix effects. Our goal is to provide an EV immunocapture approach with enough sensitivity, specificity and robustness for clinical grade diagnostic applications.
Methods: Size-based vs. immunocapture methods for exosome isolation. Enzymatic and immunological assays for plasma pre-clearing; Flow cytometry, ELISA, nanoparticle tracking analysis, Western Blot, SPR and ddPCR for antibody and exosome characterization.
Results: Exosomes derived from NSCLC cell lines display distinct membrane markers recognized by a panel of proprietary Abs, screened by flow cytometry, SPR, IP, ELISA and PCR. We developed and tested a screening platform based on endogenously labelled EVs to identify NSCLC EV antigens. Selected antibodies will be used to develop an immune-isolation protocol, coupled to state-of-the-art analytics for a rapid and sensitive readout, thus enabling a comparative evaluation of a repertoire of plasma pre-analytical protocols.
Summary/conclusion: Different plasma pre-analytical protocols are ranked and orthogonally combined to optimally counteract matrix effects, increment EV yield by immune-isolation approaches and facilitate the analysis of enriched EV subpopulations.
Funding: The project is funded under the Marie Skłodowska-Curie grant agreement No. 765,492 “ELBA – European Liquid Biopsies Academy” and internal Exosomics R&D Funds.

IP.09

Side scatter module for enhanced detection of Extracellular Vesicles by flow cytometry.

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Introduction: EVs are nanosized (20 ~ 5000 nm) membrane vesicles released from cells that can transport cargo – including miRNA and proteins – between cells as a powerful way of intercellular communication. Currently, flow cytometry is the only high throughput technique capable of single particle cell surface phenotyping and sorting with the possibility of concentration determination. Unfortunately, the drawback of standard flow cytometry is lack of sensitivity to detect smallest particles, especially for those with a size less than or equal to the dimensions of the excitation laser wavelength.
Methods: BD has developed an accessory side scatter (SSC) module for enhanced scatter detection of small particles by flow cytometry: the SP SSC module. The SP SSC module should be used in combination with a laser power of at least 100 mW. Small particle detection enhancement is achieved by significantly increasing the signal-to-noise ratio of the SSC.
Results: The SP SSC module can be installed on most commercially available BD flow cytometers, which have sufficient laser power, as an additional option. The normal SSC detector remains in place and the SP SSC module has minimal impact on regular SSC and fluorescent performance therefore use of the system for cell analysis applications is still possible. Initial results using the SP SSC module were obtained using a BD FACSCelesta™ SORP and a BD FACSAria™ Fusion, respectively having a 100 and 200 mW 488 laser. Side-by-side comparison of the regular SSC detection vs. SP SSC detection was done using polystyrene beads, silica beads, EV reference material and antibody-stained EV material.
Summary/conclusion: Utilization of the SP SSC module for sorting of natural (plasma EVs) and artificial

(liposomes) membrane particles is currently being undertaken.

IP.10

Quantitative imaging and phenotyping of EVs with 20 nm resolution

Andras Miklosi, Zehra Nizami, Blanka Kellermayer and Mariya Georgieva

ONI (Oxford Nanoimaging ltd)

Introduction: Complex extracellular vesicle (EV) phenotyping is a major technical challenge that hinders clinical translation. Single-molecule localization microscopy (SMLM) is a Nobel-Prize winning technique that allows quantitative imaging below the diffraction limit necessitating only simple and fast sample preparation. The data presented here constitutes one of the first accounts of single-molecule imaging used to successfully resolve the structure, protein (CD9, CD63, and CD81) and nucleic acid content of EVs with 20 nm resolution.

Methods: EV isolation was performed from keratinocyte culture media. EV suspensions were stained using fluorescently labelled primary antibodies raised against known exosome markers, and commercially available membrane and nucleic acid labels. Characterization of the molecular content and structural properties of surface-immobilized EVs was performed using the SMLM mode of the ONI Nanoimager. Sizing of EVs in solution was performed using the dual-colour single-particle tracking mode of the ONI Nanoimager.

Results: Multicolour super-resolution microscopy imaging of purified EVs revealed the phenotypic and structural properties of hundreds of individual vesicles at a time. The membrane staining allowed to visualize EVs with sizes ranging from ~20 nm to >250 nm, and sizing by tracking confirmed this distribution and a mean size of 120 nm. For EVs of >40 nm the membrane appeared as a ring and was a confirmation of their intact structure. CD63, CD9 and CD81 co-localized with the membrane staining at the nm scale, thus allowing to determine the molecular ID of EV subpopulations and correlate the protein marker levels with the size of EVs.

Summary/conclusion: The quantitative nature of single-molecule imaging and tracking significantly improves EV characterization. This work provides evidence of the use of SMLM imaging as a novel and powerful tool for rapid and multiplexed EV characterization with unique combination of structural and phenotypic insight.

IP.11

Benchmarking of established exosome isolation methods (density gradient centrifugation, size-exclusion chromatography and immune-bead separation) with glycan recognizing EXÖBead

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Introduction: Exosomes are small vesicles (30–150 nm) found in various human biofluids, such as plasma. OptiPrep density gradient centrifugation (DGC) is widely accepted as a pure exosome isolation method. Size-exclusion chromatography (SEC) is a fast exosome isolation method, but exhibit contaminations such as lipoprotein or aggregated proteins. Immunobeads (HBM) are based on high specific recognition of exosome CDs, but uses a harsh elution procedure to get intact exosome. EXÖBead (Biovesicle) are glycan recognition magnetic beads and show high exosome specificity by FACS, NTA and TEM analysis. In this study, we compared these four isolation methods based on FACS established exosomal markers, intact exosome size/number and lipoprotein contamination.

Methods: Mix plasma samples were collected from healthy donors (n = 5) and patients undergoing coronary angiography (n = 6). Exosomes were isolated from 250 µl plasma by SEC and DGC, fractions were collect from SEC (7 ~ 10) or DGC (6 ~ 8), and then covalent-coated on 1 µm magnetic beads (followed Chemicell). We also covalent-coated 1 ml 10% exosome free (EF) FBS in PBS as a negative control. We directly incubated 250 µl plasma with 1 µm glycan recognition magnetic beads EXÖBead (37°C, 1 h) or 1 µm latex HBM immunobeads (4°C, 16h). As a negative control 1 ml (EF) FBS was incubated. Universal antibody mix (PE-Cy7-CD63, FITC-CD81 and APC-CD9) was used for all isolation methods. The negative control reduced fluorescence data are presented by median fluorescence intensity (MFI). NTA data were collected only from intact exosomes.

Results: EXÖBead represents highest MFI of CD63 (247.9) compared to SEC (232.42), DGC (25.72) and HBM (5.13). EXÖBead also showed highest MFI of CD9 (475.4) compared to SEC (42.3), DGC (5.1) and HBM (0). Only SEC (88.9) and EXÖBead (41.1) could detect CD81. Experiment processing time for EXÖBead is 2h, SEC is 4h, HBM is 19h, and DGC even 22h. SEC represents highest intac

t exosomes/ml (4.9E+10), EXÖBead (1.7E+9), HBM (1.9E+8), and DGC (1.5E+8), measured by NTA.

Median exosome sizes are EXÖBead 72.0 nm, SEC 107.0 nm, DGC 89.6 nm and HBM 96.1 nm.
Summary/Conclusion: EXÖBead serves as a new time-saving plasma isolation method with high exosome yield and specificity.

IP.12

Characterizing the cellular uptake of neural stem-cell derived exosomes using live-cell imaging techniques

Samuel Jones[a], Thomas Caws[b], Anthony Hayes[a], Victoria Marsh Durban[b], Randolph Corteling[b] and Peter Watson[a]

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Introduction: Neural stem cell derived exosomes ("ExoPr0"); purified from the conditioned medium of a GMP manufactured, conditionally-immortalized human neural stem cell line ("CTX0E03"), demonstrates a unique biodistribution profile in mice compared to exosomes derived from a control producer cell line. We have previously shown that ExoPr0 is able to cross the blood brain barrier, and to further explicate these findings, we investigated the uptake of ExoPr0 at the cellular level using live-cell imaging techniques.
Methods: We employed live-cell confocal microscopy to directly visualize uptake of fluorescently labelled exosomes. A quantitative image analysis protocol was developed and applied to assess the uptake of exosomes in a number of cell types.
Results: Time course incubations of cells treated with ExoPr0 produced data that revealed heterogeneity in uptake between cell types. ExoPr0 was compared to exosomes derived from a control producer cell line, highlighting source-specific differences in uptake kinetics. Uptake was observed to occur through more than one pathway resulting in trafficking through endo-lysosomal compartments. The effect of cell cycle on the uptake of ExoPr0 was investigated, but was not observed as having a significant influence.
Summary/conclusion: Findings from this study have eluded to the specificity of ExoPr0 towards different cell types and work is ongoing to further elucidate the delivery mechanism of ExoPr0 and understand the subcellular trafficking in recipient cells.

Symposium Session 7: Advances in EV Isolation in Cancer
Chairs: Leonora Balaj; Johan Skog
Location: Level B1, Hall A

17:00–18:00

OT07.01

Aggregation-induced emission probe/graphene oxide aptasensor for label-free and "turn-on" fluorescent aptasensor for cancerous exosomes

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Introduction: Exosomes are the smallest subset (30–150 nm) of extracellular vesicles (EVs), a heterogeneous population of vesicles originate from all types of tissue cells, which can freely pass through the blood vessel wall and distribute in various body fluids. Exosomes carry different macromolecules, such as nucleic acids, proteins and lipids for intercellular communication. In the last decade, numerous researches demonstrated that exosomes' cargo is affected in the progression of malignant tumours, positioning exosomes as potential sources for the discovery of novel biomarkers. For example, it is confirmed that PSMA is enriched in the membrane of exosomes from prostate cancer cells. So, PSMA positive exosomes subpopulation is regarded as the diagnostic biomarker for prostate cancer. But conventional methods can hardly quantify low-concentration PSMA positive exosomes subpopulation in small volumes of clinical samples rapidly.

Methods: In this work, we constructed the label-free and "turn-on" aptasensor for the detection of the PSMA positive prostate cancer exosome based on PSMA aptamer as the recognition element, Aggregation-Induced Emission (AIE) probes: TTAPE as fluorescent indicators and Graphene Oxide (GO) as fluorescent quencher. In the absence of PSMA positive exosomes, the fluorescence of TTAPE aggregated in the aptamer would be quenched efficiently by GO. However, in the presence of PSMA positive exosomes, the specific and stronger binding between aptamers and PSMA positive exosomes could weaken the binding interaction between aptamer and GO. So the fluorescence of TTAPE aggregated in the aptamer would recover, which could appear "turn-on" fluorescent property.

Results: Under optimal conditions (37°C, 15 min), the linear range of detection for prostate cancer exosomes is estimated to be 4.07×10^5–1.83×10^7 exosomes/μL with a detection of limit (LOD) of 3.43×10^5 exosomes/μL. We further successfully applied it for exosomes quantification in plasma samples from prostate cancer patients.

Summary/Conclusion: This aptasensor is expected to become a powerful tool for rapid and simple cancer liquid biopsy.

Funding: This study was financed by grants from the National Natural Science Foundation of China (81371901, 81702100), the Science and Technology Planning Project of Guangdong Province (2017A020215123).

OT07.02

Single extracellular vesicle (EV) profiling and EV subpopulation analysis of cancer related EVs in human plasma

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Introduction: Extracellular vesicles (EV) carry important information of their parental cells, and are therefore promising biomarkers for liquid biopsy and early diagnosis of multiple diseases including cancer. However, the detection of disease specific EV among huge numbers of EVs in the clinical sample, e.g. plasma remains a challenge, which makes single EV and EV subpopulation analysis preferable to bulk analysis.

Methods: In the presented work, in order to recognize the cancer cell line specific EVs, we utilized a proximity barcoding assay (PBA) to analyse the surface protein composition of single EVs and investigated the EV subpopulation. A pool of hundred-plex oligonucleotide-conjugated antibodies against reported cancer biomarkers candidates was employed to recognize the surface proteins of individual EVs. Then all the oligonucleotides on the same EV obtained an unique EV tag in a PBA. The pool of extension products can be amplified and sequenced by next generation sequencing. After sorting the reads, we could reconstruct the surface protein composition of individual EVs.

Results: We applied PBA to analysed EVs purified from cancer cell lines and from human plasma. We could identify different subpopulation EVs, that are specific for certain cell lines and human plasma. We then spiked in different amount cancer cell-line derived exosomes in the plasma derived EVs from healthy donors in different ratio. We could observe en expected increase of certain population of exosomes in the human plasma.

Summary/Conclusion: In summary, PBA is a multiplexed and high throughput approach to analyse surface proteins of individual EVs. The cancer cell line EVs mixed into healthy control plasma were successfully detected, indicating this technique can be applied to search for rare population of EVs in the plasma samples of patients.

Funding: National Natural Science Foundation of China, project 81802052

OT07.03

miRNA signature derived from GBM plasma exosomes as a diagnostic biomarker

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Introduction: Gliomas including glioblastoma (GBM) are the most common malignant brain tumours. Glioma extracellular vesicles (EVs), especially plasma exosomes, have biological effects such as mediating immunosuppression and contain signature tumour-specific cargo that could serve as liquid biopsies. Increasing interest in molecular biomarkers to determine patient prognosis in GBM has suggested that EV miRNA-based signatures may be able to predict progression-free and overall survival, differentiate normal donors from GBM patients, and distinguish true progression from treatment-related pseudo-progression.

Methods: We have established a simple technique, using density gradient ultracentrifugation, to isolate plasma exosomes from glioma patients and normal donors. Purification of total RNA, including miRNA, was performed on plasma exosomes from normal donors (n = 8) and GBM patients (n = 7) using the miRNeasy kit (Qiagen). Next generation short non-coding RNA sequencing was performed by Illumina HiSeq 4000.

Results: RNA sequencing revealed many differentially expressed miRNAs in GBM patients with high fold change/low false discovery rates compared to normal donor plasma exosomes. Ingenuity Pathway Analysis showed that these differentially expressed miRNAs target mRNAs that are associated with distinctive GBM and cancer pathways. In order to test the diagnostic accuracy of the proposed technique, ROC analysis was performed based on the top 33 differentially expressed miRNA samples. The area under the ROC curve (AUC; a figure of merit to determine the optimal miRNA signature) was 0.968. In addition, multiple novel miRNAs and other short non-coding RNA species (Y-RNA, piRNA, snoRNA) were found with some differential expression.

Summary/Conclusion: In conclusion, miRNA sequencing from plasma exosomes shows marked differential miRNA expression between healthy donors and GBM patients. These findings as well as additional differentially expressed short non-coding RNA species suggest plasma EVs may serve as a robust platform to develop GBM liquid biopsies.

Funding: Mayo Clinic Center for Individualized Medicine (CIM) Brains Together For a Cure

OT07.05

Isolation of extracellular vesicles by nanoDLD lab-on-a-chip technology for clinical applications

Stacey M. Gifford[a], Joshua Smith[a], Benjamin Wunsch[a], Navneet Dogra[a], Mehmet Ahsen[b], Kamlesh Yadav[c], Ashutosh Tewari[d], Carlos Cordon-Cardo[e] and Gustavo Stolovitzky[a]

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Introduction: There is great interest in exosome isolation and analysis to develop non-invasive "liquid biopsies" for diagnosis, prognosis, and surveillance of diseases. However, current exosome isolation methods lack purity, yield and reproducibility and the inability to rapidly and reliably separate exosomes hinders clinical application. Thus, there is an urgent need to develop novel tools to isolate exosomes as a promising source of new biomarkers.

Methods: We have developed a lab-on-a-chip technology based on deterministic lateral displacement at the nanoscale (nanoDLD) which separates and concentrates particles in continuous flow and in specific size ranges, going to scales as small as 20 nm. We used nanoDLD to isolate EVs from urine and serum and characterized these EVs by NTA and RNA sequencing.

Results: Benchmarking studies of nanoDLD isolation of exosomes show comparable or improved yield and concentration compared to standard techniques such as SEC and UC at volumes suitable for clinical applications. We isolated EVs from the urine and serum of prostate cancer (PCa) patients. Our preliminary data show PCa patient serum exosomes are enriched in known PCa biomarkers. Screening for an EV RNA panel associated with aggressiveness could aid detection of clinically significant PCa and reduce unnecessary radical prostatectomies.

Summary/Conclusion: We have developed a chip-based tool for EV separation and demonstrated improved yield, concentration and processing time compared to existing isolation methods. This technology has enabled high-resolution temporal studies of urinary EVs to better understand the impact of pre-analytical challenges on EV studies. Finally we used nanoDLD to isolate EVs from prostate cancer patient samples and detect an enrichment of known mRNA prostate cancer markers in serum EVs. Our nanoDLD technology enables frequent, rapid isolation of EVs at improved yield and concentration enabling the use of smaller sample volumes.

Funding: Work was funded by IBM Research and the Icahn School of Medicine at Mount Sinai.

Symposium Session 8: Mechanisms of Delivery
Chairs: Lorraine O'Driscoll; Carlos Salomon
Location: Level 3, Hall B

17:00–18:00

OT08.01

Magnetically navigated intracellular delivery of extracellular vesicles using nanogels

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Introduction: Extracellular vesicles can control important biological phenomena such as cell differentiation and cell death. In addition, extracellular vesicle is also regarded as a promising material for biomedical application. However, due to their low efficiency of intracellular uptake, development of effective intracellular delivery method has been remained challenging issue. We report here the complexation of extracellular vesicles and magneto-responsive nanogels, and efficient intracellular delivery of extracellular vesicles into cells by magnetic guidance for induction of differentiation of stem cells by delivered extracellular vesicles.
Methods: Magnetic nanogels were prepared by mixing oleic acid-coated iron oxide nanoparticles dispersed in an organic solvent to nanogels composed of cholesteryl group-substituted pullulan. Magnetic nanogel-exosome complexes were prepared by isolating exosomes from culture supernatants of myoblasts and nerve cells by ultracentrifugation and mixing this exosome with magnetic nanogels. The resulting magnetic nanogel-exosome complex was delivered to the cells by magnetic induction and its intracellular dynamics were investigated using a confocal laser microscope and flow cytometry.
Results: In 24 h, 90% of exosome could be complexed with magnetic nanogel. The obtained magnetic nanogel-exosome complex was delivered to adipose-derived mesenchymal stem cells (ADSC) by magnetic induction. As a result, the introduction of magnetic nanogel and exosome into the cytoplasm was confirmed. From the results of immunostaining, expression of the differentiation marker was confirmed in which the complex was introduced to ADSC by magnetic induction for both myoblasts and nerve cells.
Summary/Conclusion: Differentiation was induced to ADSC by efficient magnetic delivery of exosome. This magnetic nanogel introduction method is expected to be used as analysis of exosomes whose function is unclear or as a novel cell function control technique using exosome.

OT08.02

Tissue distribution of extracellular vesicle-binding proteins after in vivo gene transfer into mice

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Introduction: Successful application of extracellular vesicles (EVs) as delivery systems for bioactive molecules, including miRNAs and tumour antigens, requires the understanding and control of their tissue distribution. Our previous studies demonstrated that the exogenously administered EVs of about 100 nm in diameter quickly disappeared from the systemic circulation after intravenous injection into mice. Despite these results, endogenous EVs might have different tissue distribution properties from exogenously administered ones. To test this hypothesis, it is important to develop a method to analyse the properties of endogenous EVs. In this study, as a first step, we selected *Gaussia* luciferase (gLuc) and lactadherin (LA) as a reporter protein and an EV-binding protein, respectively, and examined whether the fusion of LA to gLuc could alter the tissue distribution of gLuc after *in vivo* gene transfer into mice.
Methods: pcDNA3.1 plasmid vectors encoding gLuc, a fusion protein of gLuc and LA (gLuc-LA), or a fusion protein of gLuc and a mutated LA which has low affinity to EVs (muLA) were constructed (pCMV/gLuc, pCMV/gLuc-LA and pCMV/gLuc-muLA). Each plasmid was injected into 4-week-old male ddY mice using the hydrodynamic injection method, and blood was collected at several time points to obtain plasma. Then, EVs in plasma were separated and collected by the ultracentrifugation method. The characteristics of the EVs were evaluated by western blotting and dynamic light scattering. The luciferase activity of the plasma and the EVs was measured in a luminometer.
Results: In all the cases examined, the luciferase activity in the plasma was very high soon after

hydrodynamic injection of the plasmid vectors, then it decreased with time. No significant luciferase activity was detected in the EVs when pCMV/gLuc or pCMV/gLuc-muLA was injected. By contrast, about 5% of luciferase activity of the plasma was recovered in the EV fraction when mice received an injection of pCMV/gLuc-LA.

Summary/Conclusion: These results indicate that gLuc-LA binds to EVs in mouse blood through LA after *in vivo* gene transfer, which suggests that gLuc-LA can be used to analyse the tissue distribution of endogenous EVs.

OT08.03

Capabilities of HEK293T cell-exosomes as a non-invasive delivery tool for mammalian sperm

Teresa Vilanova[a], Celine Jones[a], Rebecca Dragovic[a], Kevin Coward[a] and Marc Yeste[a]

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Introduction: Male infertility accounts for 35–50% of human infertility, and 2.5–12% of men present some form of infertility. Several non-invasive approaches to treat sperm-borne aberrations are being developed such as exosomes for compound delivery. Human Embryonic Kidney (HEK)293T cell-exosomes appear to be safe and versatile in terms of their targeting abilities. However, the safety aspects for gametes need to be investigated. In this study we developed HEK293T cell-exosomes for *in vitro* co-incubation with boar sperm. Exosome binding and exposure effects (for viability, mitochondrial membrane potential (MMP) and membrane fluidity (MF)) were examined.

Methods: HEK293T-exosomes were characterised by Nanoparticle Tracking Analysis, Western Blotting and Transmission Electron Microscopy. Boar sperm samples (n = 3) were in vitro co-incubated at an exosome: sperm ratio of 10:1 (4h pH<7). Sperm aliquots at 0, 2 and 4h post-incubation were analysed for exosome binding. Moreover, boar sperm (n = 5) was *in vitro* co-incubated at different ratios (1:1, 10:1 and 100:1) under capacitating and progesterone-induced hyper-activating conditions. Analysis at 0h, 2h, 4h, 4h 10 min, 4h 30 min and 5h post-incubation by flow cytometry for viability, MMP and MF of exosome-treated samples was performed by staining with SYBR-14/PI, JC-1 and YO-PRO-1/Merocyanine-540, respectively. Data were analysed with a mixed model (between-subjects factor: treatment; within-subjects factor: incubation time) followed by the post-HOC Sidak test.

Results: Data revealed an homogeneous exosome-enriched sample in terms of exosome-like morphology and size. Exosome-sperm binding to the head, mid-piece and tail was confirmed with up to two exosomes/sperm cell. No statistically significant differences were found in terms of viability, MMP and MF for any of the tested ratios at each time point, compared to controls.

Summary/Conclusion: HEK293T cell-derived exosomes bound to all sperm parts soon after the incubation began. A high exosome concentration did not compromise the viability nor the response of boar spermatozoa to induced capacitation and acrosome-exocytosis in vitro. In conclusion, HEK293T cell-exosomes have shown to have potential as a future clinical delivery system in the context of male infertility.

Funding: SRF and St. Peter's College (University of Oxford).

OT08.04

Extracellular vesicles from de-differentiated human adipose tissue endothelial cells have potential to disseminate angiostatic signals in human obesity

Anca D. Dobrian[a], Bronson Haynes, Ryan Huyck, Lifang Yang, Vanessa Correll, William McPheat and O. John Semmes[b]

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Introduction: Endothelial-to-mesenchymal transition (EndoMT) characterized by endothelial cell (EC) de-differentiation into a mesenchymal phenotype is a focal event present in the vasculature of obese adipose tissue (AT) and has been shown to contribute to various vascular pathologies. EC from human AT impacted by EndoMT are angiostatic and have a quiescent metabolic phenotype. We hypothesize that extracellular vesicles (EV) produced by such EC may lead to propagation of angiostatic signals which may contribute to hypoxia and insulin resistance in obese AT.

Methods: We modelled EndoMT in vitro by treatment of human AT ECs with pro-inflammatory cytokines and prepared EV from conditioned media by ultracentrifugation. Uptake of EVs by naïve EC was measured by flow cytometry; angiogenesis by in vitro tube formation; and mitochondrial energetics with Seahorse bioanalyzer. The miRNA cargo of the EVs was analysed using the Nanostring platform and the proteome was determined using LC/MS/MS.

Results: EV from EndoMT cells produced a dramatic angiostatic effect on recipient EC without affecting migration or proliferation. Recipient EC became quiescent and had lower ATP production compared to controls. Pathway analysis of EV cargo showed significant

targeting of fatty acid synthesis and oxidation in recipient EC. We found abundant miR-155-3p in EV and reduced expression of its metabolic enzyme targets CPT1a and ACLY in recipient EC. Treatment of EC with the CPT1a inhibitor etomoxir recapitulated the angiostatic effect of the EVs. The EV proteome was also enriched in peptide signatures for VEGFR1, VEGFR2 and neuropilin.

Summary/Conclusion: We show that the metabolic shift produced by EV from EndoMT cells may explain their angiostatic effect. miR-155 delivered via EV may be key for metabolic quiescence via inhibition of CPT1 and ACLY. We report a novel mechanism by which EndoMT in EC produces EVs that may propagate angiostatic effects throughout the AT vasculature in obesity.

Funding: NIHR15NHLBI, American Heart Association-AIREA.

Symposium Session 9: EV Biogenesis II
Chairs: Bong Hwan Sung; Graca Raposo
Location: Level B1, Hall B

17:00–18:00

OT09.01

Different exosome subtypes have distinct ESCRT-associated biology and control tumour cell adaptation in vivo

Shih-Jung Fan[a], Benjamin Kroeger[b], Pauline Marie[a], Esther Bridges[a], Kristie McCormick[a], John Mason[a], Helen Sheldon[a], Claudia Mendes[a], Mark Wainwright[a], John Morris[a], Adrian Harris[a], Clive Wilson[a] and Deborah C I. Goberdhan[a]

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Introduction: Determining the function of specific extracellular vesicle (EV) and exosome subtypes has proved challenging, in part due to the difficulty in untangling the mechanisms leading to their generation.
Methods: We investigated the cell biology behind exosome formation using the large endosomal compartments offered by an *in vivo* fly model, and analysis in human HCT116 and other cancer cell lines. EV preparations were also tested in vivo following injection in to human xenografts in mice. We analysed different EV preparations by mass spectrometry using Tandem Mass Tag labelling to identify changes in protein cargo of EVs in response to microenvironmental stress.
Results: Using these complementary approaches, we show that microenvironmental stress, such as glutamine depletion, leads to a switch in membrane trafficking from the classic late endosomal multivesicular endosomes to Rab11a-positive recycling endosomes and the production of Rab11a-positive exosomes, which promote cell growth under stress conditions. This activity is suppressed by blocking Rab11a-dependent trafficking and ESCRT function. Our proteomics and fly data suggest that some ESCRTs are differentially involved in these two exosome-generating processes. Furthermore, mouse xenografts highlight roles for stress-induced EVs in increasing the turnover of tumour cells, leading to an increase in hypoxic stress, associated with selection for aggressive cells that can promote tumour progression. These stress-induced vesicles also have a potent effect on blood vessel growth *in vivo*.
Summary/Conclusion: We conclude that stress-induced EVs and exosomes made in Rab11a-positive recycling endosomes are involved in tumour adaptation.

Funding: This work was funded by Cancer Research UK [C19591/A19076], the CRUK Oxford Centre Development Fund [C38302/A12278], BBSRC [BB/K017462/1, BB/N016300/1, BB/R004862/1], John Fell Fund, Oxford, Wellcome Trust [MICRON; #091911, #107457], Royal College of Surgeons.

OT09.02

Emerging role of L-type calcium channel-mediated calcium influx in regulating apoptotic bodies formation

Thanh Kha Phan[a], Bo Shi[b], Niall Geoghegan[c], Kelly Rogers[d] and Ivan Poon[e]

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Introduction: Dying cells often break into smaller membrane-bound fragments, called apoptotic bodies (ApoBD), via apoptotic cell disassembly (ACD), an essential physiological or pathophysiological event downstream of apoptosis. Emerging evidence implies the importance of ApoBD formation in mediating efficient phagocytic removal of apoptotic debris and facilitating intercellular communication through trafficking of biomolecules and pathogen-derived materials. In contrast to long-lasting belief, our recent findings have demonstrated that apoptotic cell disassembly is a tightly regulated and temporally-controlled three-step process: (i) membrane blebbing, (ii) formation of thin membrane protrusion promoting bleb separation and (iii) protrusion fragmentation to form ApoBD. However, detailed insights to the underlying mechanism, particularly ion channels and chemical signalling, undoubtedly require further investigations.
Methods: To identify ion channel(s) involved in ACD process, cells were treated channel blockers prior to UV irradiation. ApoBD formation was monitored using DIC microscopy and quantified by our recently-developed multi-parametric flow cytometry analysis using TOPRO-3 dye and Annexin V. Lattice light sheet microscopy allowed us to obtain high-resolution imaging of calcium-mediated ACD in presence of various fluorescent stains.

Results: Our data showed that calcium influx preceded disassembly step of apoptotic cell, blockade of which, using calcium channel inhibitors, abolished ApoBD formation. Strikingly, calcium channels contain a tentative caspase cleavage site, immediately preceding calmodulin-binding IQ motif which mediates calcium-dependent feedback inactivation of the channels. Thus, maximised calcium influx by caspase-cleaved calcium channels could be a novel regulatory mechanism of ACD. Furthermore, we could monitor the detailed progression of the process, from cytosolic calcium accumulation to form electrochemical force, driving protrusion formation and ACD process.
Summary/Conclusion: Our findings therefore provide further molecular insights into dying cell disassembly and calcium-induced ApoBD-associated pathogenesis, particularly vascular calcification.

OT09.03

A novel UBL3 modification influences protein sorting to small extracellular vesicles

Hiroshi Ageta[a], Natsumi Ageta-Ishihara[b], Keisuke Hitachi[a], Takanori Onouchi[a], Hisateru Yamaguchi[a], Yusuke Yoshioka[c], Nobuyoshi Kosaka[d], Tomihiko Ide[a], Makoto Kinoshita[b], Takahiro Ochiya[d], Mitsutoshi Setou[e] and Kunihiro Tsuchida[a]

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Introduction: Small extracellular vesicles (sEVs) are nanometre-sized vesicles secreted from various cell types. Exosomes, a type of sEVs, derived from multivesicular bodies (MVBs), mediate cell-to-cell communication by transporting proteins, mRNAsand miRNAs. The delivery of proteins between cells by sEVs, including exosomes, is related to tumour progression and neurodegenerative diseases. However, the molecular mechanism by which proteins are sorted to sEVs is not fully understood.
Methods: By using immunoprecipitation, immunocytochemical, electron microscopic and proteomics analysis, we report that ubiquitin-like 3 (UBL3)/ membrane-anchored Ub-fold protein (MUB), an evolutionarily conserved protein, acts as a novel posttranslational modification (PTM) factor that regulates protein sorting to sEVs.
Results: We find that UBL3 modification is through cysteine residues only under non-reducing conditions and is indispensable for sorting of UBL3 to MVBs and sEVs. Furthermore, we observe a 60% reduction of total protein, but not RNA, levels in serum sEVs purified from UBL3-knockout (KO) mice compared with those from wild-type mice. To identify the types of proteins that are modified by UBL3, we perform comprehensive proteomics analysis and find 1,241 UBL3-interacting proteins depending on the two C-terminal cysteine residues. Among these, 369 proteins are annotated as “extracellular vesicular exosome” by Gene Ontology (GO) analysis, and there are at least 22 disease-related molecules, including Ras. To investigate whether UBL3 modification affects protein sorting to sEVs, we choose Ras as a model protein. We show that Ras and oncogenic RasG12V mutant are post-translationally modified by UBL3, and that increased sorting of RasG12V to sEVs by UBL3 modification enhances activation of Ras signalling in the recipient cells.
Summary/Conclusion: Collectively, these results indicate that a novel PTM by UBL3 influences the sorting of proteins to sEVs. UBL3 modification could be a novel therapeutic target for sEV-related disorders.

OT09.04

Stringent small extracellular vesicle purification and ligation-independent small RNA-seq: new insights into released RNA populations

Kenneth W. Witwer[a], Tine Schøyen[a], Yiyao Huang[a], Andrey Turchinovich[b], Senquan Liu[a], Linzhao Cheng[a] and Vasiliki Machairaki[c]

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Introduction: MicroRNAs are a major focus of exRNA and EV studies. Many publications report miRNAs as the plurality or majority of released small RNAs. However, legacy sRNA profiling methods are biased towards miRNAs. Abundant RNAs outside vesicles also contaminate many EV preparations. We sequenced exRNA from induced pluripotent stem cells (iPSCs) with a ligation-independent method: ultra-low-input capture and amplification by tailing and sequencing (CATS).
Methods: Culture conditioned medium (CCM) was collected from 4 lines of count-normalized iPSCs over 3 passages (> 200 mL/passage). Fractions were: cells (washed/lysed); “whole releasate" = clarified CCM (300 x g, 2k x g); “large EVs (lEVs)" = pellet of 10k x g spin; “small EVs (sEVs) = preparation by tangential flow filtration (100 kDa cutoff) and size exclusion chromatography (Izon); and “soluble" = flow-through from sEV preparation. Particles were counted by ParticleMetrix, visualized by TEM, and tested for up to 7 positive or negative markers per MISEV2014/18. lEVs and sEVs were treated with nucleases. CATS sRNA libraries were analysed for contribution of

RNA classes. Statistics were corrected for multiple comparisons; significance = corrected $p < 0.01$.

Results: Using CATS, miRNAs mapped at only a small % of total sRNA reads; typically less than 1%. Nuclease-treated sEVs had significantly lower relative miRNA levels than cells or soluble releasate. tRNAs/fragments had highest relative abundance in whole releasate and soluble fractions, albeit with substantial variability. Significantly different in most releasate fractions vs cells were sno/scaRNA, mRNA, and lncRNA. Cellular distribution differed only from lEV and sEV for RNU RNAs, and only from sEV for Y RNAs. rRNAs/fragments did not differ significantly. Medium-only process controls had only a small per cent of human mapping.

Summary/Conclusion: miRNAs are found at lower relative levels in cells and releasate than indicated by legacy sequencing methods. miRNAs also tend to be excluded from sEVs vs. cells or other releasate fractions. While this study uses iPSCs, similar results would likely be obtained with other cells. We do not discount the role for miRNAs in cell-cell communication but suggest that sEVs may not be a vastly superior source of miRNAs.

Funding: This work was supported by the US NIH: NIA (AG057430), NIDA (DA040385 and DA047807) and NIMH (MH118164).

Symposium Session 10: EVs in Blood and Blood Disorders
Chairs: Ai Kotani; Rienk Nieuwland
Location: Level B1, Lecture Room

17:00–18:00

OT10.01

Different ATT isoforms are associated to EVs from ATT type II deficient patients

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Introduction: Antithrombin (AT) is a glycoprotein involved in the regulation of blood coagulation. It belongs to the family of serine-protease inhibitors and acts as the most important antagonist of different clotting factors. Two types of inherited AT deficiency can be distinguished: Type I (quantitative deficit), and Type II (qualitative deficit). The latter is characterized by an impaired inhibitory activity related to dysfunctional domains of the protein. Three Type II subtypes can be defined: Type IIa (reactive site defect), Type IIb (heparin binding site defect) and Type IIc (pleiotropic defect). This classification has clinical importance since these subtypes have a different thrombotic risk. No functional routine diagnostic assay, however, can be assumed to detect all forms of Type II deficiencies since false-negative results may hamper the diagnosis.
Methods: We analysed the biochemical/biophysical association of ATT to EVs. We separated EVs from plasma of healthy or Type II affected patients or from cultured hepatocytes through differential ultracentrifugation followed by sucrose density gradient and/or immunoprecipitation. We next combined dot blot analysis, WB, 2D electrophoresis and enzymatic assays to reveal the nature of ATT association to EVs.
Results: We evidenced that ATT is associated to the external leaflet of EVs. We also found that specific ATT isoforms are enriched in EV preparations in respect to total plasma and that those isoforms are selectively associated to EVs when comparing healthy or ATT type II deficient patients.
Summary/Conclusion: ATT selective association pattern to EVs might be related either to mutations in the primary sequence of the protein or alterations in the glycosylation process, hence experiments are ongoing to reveal the nature of this phenomenon. Our findings suggest that analysis of ATT enriched in EV preparations might be useful to gain insights into the pathogenesis and be of support in the diagnostic algorithm of ATT deficiency.
Funding: This work acknowledges FFABR (Fondo finanziamento attività Base di ricerca from MIUR, Ministry of Education, Universities and Research, Italy) for financial support.

OT10.02

Search for EV signature in sickle cell disease

Sisareuth Tan[a], Celine Gounou[a], Marc Romana[b], Stephane Mornet[c], Alain R. Brisson[d]

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Introduction: Sickle cell disease (SCD) is a hereditary haemoglobinopathy characterized by the production of sickled red blood cells (RBC), anaemia and vascular occlusion crises. The presence of extracellular vesicles (EV) in blood from SCD patients has long been recognized, yet with a large divergence of results (1). Our objective was to characterize in details EV in plasma from SCD patients, by combining flow cytometry and immuno-gold cryo-electron microscopy (2,3). We focused on two EV populations: 1) EV exposing phosphatidylserine (PS), because the increased exposure of PS at the RBC surface is a hallmark of SCD (4), and 2) exosomes exposing CD71 (CD71-Exo), because the reticulocyte count is a marker of anaemia and CD71-Exo are released during the maturation of reticulocytes into erythrocytes (5).
Methods: Platelet-free plasma (PFP) was obtained from 11 SCD patients and 18 control individuals. Annexin-5, anti-CD235a- and anti-CD71-IgGs, either fluorescently labelled or conjugated to gold particles, were used to detect PS+ EV, RBC-derived EV and CD71-Exo, respectively, by flow cytometry and immuno-cryo-EM (2,3).
Results: By flow cytometry, seven populations of RBC-derived EV were identified in SCD plasma, based on the presence vs. absence of PS, EV size and morphology. The main difference between SCD and control

PFP was the presence in SCD PFP of large amounts of PS+ EV of small size (100 to 200 nm, as determined by immuno-cryo-EM) (250,000 ± 20,000 / µL for SCD PFP vs. 30,000 ± 10,000/µL for control PFP).

In addition, CD71-Exo were detected in SCD PFP by immuno-cryo-EM, while they are almost absent in control PFP. As expected, CD71-Exo were highly homogeneous in size, ranging from 50 to100 nm. Their concentration was determined by fluorescence-triggered flow cytometry: 70,000 ± 40,000 / µL for SCD PFP vs. 7,000 ± 5,000 / µL for control PFP.

Summary/Conclusion: We have identified two EV populations present in large amounts in SCD plasma, while they are almost absent in control plasma. Further study is needed to evaluate the use of these EV as biomarkers of the coagulation or endothelium activation states in SCD.

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2. Arraud et al., J. Thromb Haemost 2014 12:614
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Funding: Labex GR-Ex

OT10.03

Surface protein cargo of extracellular vesicles in blood plasma; the effect of an inflammatory disease on the vesicle surface protein interactome

Eszter Á. Tóth, Katalin É. Szabó-Taylor, Tamas Visnovitz, György Nagy and Edit I Buzás

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Introduction: Extracellular vesicles (EVs) are endogenous nanoparticles produced by cells. Artificial nanoparticles used for targeted therapy have been found to develop a protein corona altering their biodistribution and bioavailability in biological media. Here we set the aim to study if a similar protein corona is formed at the surface of EVs in biofluids and if inflammation had an effect on the protein corona formation.

Methods: Blood plasma depleted in both platelets and EVs was generated from blood samples of healthy subjects (n = 12) and rheumatoid arthritis patients (n = 10). Nascent EVs of THP1 cells and platelets were isolated and incubated in the plasma samples for 30 minutes. EVs were then washed and were studied by mass spectrometry (MS/MS), immune electron microscopy and flow cytometry. Controls included i) plasma without the addition of EVs; ii) EVs incubated in buffer. The effect of different protein coronas was also studied by phagocytosis and TaqMan® assays. Flow cytometry was also performed after saline washing and protease digestions. All experiments were performed in accordance with the Declaration of Helsinki. Infomed consent was obtained from all participants.

Results: A significantly higher number of proteins was found in the plasma+EV samples compared with the summed number of proteins found in the only plasma and the only EV samples (p < 000.1). Subtracting the proteins that were found in pure EV samples from the list of proteins of EV samples incubated in plasma for 30 min and washed 2 times, a high number of proteins were found, out of which several were more characteristic of rheumatoid arthritis samples and only a few were more prevalent in healthy samples. Interactions between fibrinogen, haptoglobin, complement protein C3. and EVs were also confirmed by flow cytometry and immune electron microscopy.

Summary/Conclusion: Our data suggest the existence of a protein corona on EVs of blood plasma. The differences in protein coronas found between healthy controls and patients with rheumatoid arthritis suggest that EV surface-associated proteins may play a role in disease pathology and may serve as biomarkers.

Funding: NKFIH-OTKA PD 104369; PD 112058; 111958; 120237, NVKP_16, MTA-SE Immun-Proteogenomikai EV Kutatócsoport, VEKOP-2.3.2-16-2017–000002, VEKOP-2.3.3-15-2017-00016 H2020 MSCA ITN TRAIN-EV, SE STIA-2017

OT10.04

Oxidized LDL stimulates production of inflammatory extracellular vesicles by platelets

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Introduction: Platelets may become activated under hyperlipidemic conditions and are thought to promote atherosclerotic plaque development. Platelets can generate a diverse mixture of extracellular vesicles (EVs) when they are activated through different signalling pathways. In this study, we investigated in detail the EV-generating capacity of different lipoproteins and compared the cellular effects of the resulting EVs on macrophage differentiation.

Methods: Platelets (isolated by gel-filtration from fresh concentrates) were stimulated by LDL, oxidized LDL or HDL together with thrombin + collagen co-stimulus, a

potent EV-inducing signal. After careful platelet removal, EVs were isolated by serial ultracentrifugation. Platelet activation was monitored by P-selectin exposure in flow cytometry. EVs were analysed by an EV-dedicated high-resolution flow cytometer and Western blotting, and quantified by protein concentration and particle number. Macrophage differentiation was induced by GM-CSF in the presence or absence of the different EVs. After 6 days, macrophages were activated by IFN-γ and LPS, and after a further 3 days, the macrophages were profiled by flow cytometry and their secreted cytokines.

Results: Lipoproteins induced platelet EV production in a concentration- and time-dependent manner at concentration levels relevant to hyperlipidemic conditions. Oxidized LDL increased EV formation by platelets, whereas co-incubation with HDL inhibited this effect. Platelet derived EVs modulated the macrophage differentiation as seen by the changes in their pro-inflammatory cytokines and surface marker profiles.

Summary/conclusion: In conclusion, hyperlipidemic lipoprotein profiles in plasma can manifest in (1) altered platelet EV generation which in turn (2) may program macrophage differentiation in a manner relevant for atherosclerotic plaque development.

Funding: Academy of Finland grant 287089, Finnish Foundation for Cardiovascular Research

Symposium Session 11: EV Therapeutics I
Friday 26 April 2019
Chairs: Andre Gorgens; Sai Kiang Lim
Location: Level B1, Hall B

08:30–10:00

OF11.01

Exosomes from cerebral endothelial cells suppress chemotherapy-induced peripheral neuropathy and sensitize anti-tumour effects of platinum drugs

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Introduction: Platinum-based drugs are commonly used to treat cancers. However, peripheral neuropathy is a common adverse effect of platinum-based chemotherapy. Neurotoxicity often requires platinum drug dose reduction thereby, compromising therapeutic efficacy of platinum drugs to suppress tumour progression.
Methods: Using differential ultracentrifugation, we isolated exosomes from cultured human primary cerebral endothelial cells (CEC-exos). Ovarian tumour was induced in mice by implantation of human ovarian cancer cells. Platinum-induced CIPN start from distal axons. Thus, we examined the direct effect of platinum drugs on distal axons of dorsal root ganglia (DRG) neurons using a microfluidic device that separates distal axons from their parent cell bodies.
Results: We found that addition of oxaliplatin or carboplatin into the axonal compartment significantly suppressed axonal elongation, whereas application of CEC-exos into the axonal compartment completely abolished oxaliplatin-inhibited axonal growth. In vivo, treatment of tumour-bearing mice with platinum drugs (n = 7/group) induced CIPN characterized by tactile and cold allodynia, reduction of sensory nerve conduction velocity, and decreases of the number of epidermal nerve fibres compared to the control mice (n = 7/group). However, tumour-bearing mice treated with platinum drugs along with CEC-exos (n = 7/group) exhibited a significant reduction of platinum-drug induced peripheral neuropathy. Moreover, CEC-exos in combination with platinum drugs significantly decreased tumour size by 80–91% compared to platinum drugs alone which reduced tumour growth only by 50–72%. In sciatic nerve tissues, CEC-exos in combination with platinum drugs significantly increased miR-15b, −26a, and −214, and substantially reduced axonal damage protein levels of PTEN, SARM1, and TRPV1. In tumour tissues, the combination treatment significantly increased miR-15b and −26a, and reduced their target chemoresistant protein levels of P-gp and ABCC1.
Summary/Conclusion: Our data demonstrate that CEC-exos reduce CIPN by reversing the platinum-inactivated neuroprotective network and that CEC-exos suppress a chemoresistant network of miRNAs/protein-coding genes to enhance the anti-tumour effect of platinum drugs.
Funding: NIH R01CA219829; NIH R01NS088656; AHA 16SDG29860003.

OF11.02

EV-mediated *in vitro* transcribed (IVT) mRNA-based gene delivery for specific pro-drug activation in the tumour treats breast cancer in mice with no offsite toxicity

Alexis Forterre[a], Jing-Hung Wang[a], Alain Delcayre[b], Kyuri Kim[c], Carol Green[c], Mark Pegram[d], Stefanie Jeffrey[e] and Ac Matin[f]

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Introduction: We previously reported EV-mediated specific delivery of a gene (as mRNA) encoding the HChrR6 enzyme to orthotopically implanted HER2$^+$ breast cancer tumours in mice. HChrR6 activated the pro-drug CNOB and completely inhibited tumour growth. EVs and CNOB were delivered systemically. A new plasmid loaded the EVs with the mRNA; they displayed the EVHB protein containing anti-HER2 scFv ("PEVs"). However, no direct evidence was presented that the treatment was free of off-target toxicity. Here, three aspects, relevant to clinical transfer of the regimen, are explored: IVT- instead of plasmid-based mRNA loading; use of the pro-drug CB1954 whose safe

dose in humans is known (HChrR6 also activates CB1954); haematological and histopathologic studies to detect off-target toxicity.

Methods: Exogenous mRNA delivery using exosomes is difficult because of its fragmentation. To ensure the mRNA integrity (at synthesis, loading, and delivery), we used the "MCHB" test. MCHB is the product of CNOB activation and is strongly fluorescent, providing a facile method to test this. The mRNA was transcribed in vitro from the HChrR6 gene (standard kits) and was tested by translating it into the enzyme and ascertaining the enzyme's ability to produce fluorescence upon CNOB addition. mRNA copy number in the EVs and the recipient cells was determined by qRT-PCR. The loaded EVs were HER2 targeted as above, using EVHB ("IEVs"). HER2+ BT474 breast cancer tumours were orthotopically implanted in mice.

Results: While 5,000 PEVs were needed to deliver one copy of the mRNA, <50 IEVs delivered this. BT474 cells transfected in vitro with IEVs showed significantly higher mRNA expression than those transfected with PEVs. Expression of the latter peaked at ca. 72 h; of the former continued throughout the length of the experiment (120 h). We found that CB1954 (20 mg/kg) completely arrested tumour growth with 220-fold fewer IEVs (3.4 x 10^8) than PEVs; experiments in progress suggest that even lower IEVs may be sufficient. Extensive haematological and histopathologic studies showed no significant toxicity.

Summary/Conclusion: IEV + CB1954 therapy completely arrests tumour growth at a much lower IEV dose and without introducing potentially harmful plasmid DNA and off-target toxicity. The findings move this approach closer to clinical transfer.

Funding: NIH NCATS UH3TR000902.

OF11.03

High yield hMSC derived mechanically induced xenografted extracellular vesicles are well tolerated and induce potent regenerative effect *in vivo* in local or IV injection in a model of chronic heart failure

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Introduction: On the road towards the use of extracellular vesicles (EVs) for regenerative medicine, technological hurdles remain unsolved: high-yield, high purity and cost-effective production of EVs.

Methods: Pursuing the analogy with shear-stress induced EV release in blood, we are developing a mechanical-stress EV triggering cell culture approach in scalable and GMP-compliant bioreactors for cost-effective and high yield EV production. The third generation setup allows the production of up to 300,000 EVs per Mesenchymal Stem Cell, a 100-fold increase compared to classical methods, i.e physiological spontaneous release in depleted media (around 2000 EVs/cell), with a high purity ratio 1 × 10e10 p/µg

Results: We investigated *in vitro* the regenerative potential of high yield mechanically induced MSC-EVs by demonstrating an equal or increased efficiency compared to classical EVs with the same amount of EVs. The regenerative properties of mechanically induced MSC-EVs was confirmed *in vivo* in a murine model of chronic heart failure demonstrating that high, medium shear stress EVs and serum starvation EVs or mMSCs had the same effect using local injection. We later on tested the effect of the injection route and the use of xenogenic hMSC-EVs on their efficiency in the same model of murine chronic heart failure. Heart functional parameters were analysed by ultrasound 2 months (1 month post EV injection) post infarction. Interestingly, hMSC-EVs had the same effect compared to mMSC-EVs in local injection, showing that xeno-EVs in immunocompetent mices was well tolerated. Moreover, hMSC EV IV injection was as efficient as local intra-myocardium muscle injection with an increase in the left ventricular ejection fraction of 26% compared to pre-treatment values, whereas PBS injected controls lost 13%.

Summary/Conclusion: We demonstrated an equal or superior regenerative effect of high yield mechanically produced EVs compared to spontaneously released EVs or parental cells *in vitro* and *in vivo*, and good tolerance and efficacy of hMSC EV both with local and IV injection. This unique technology for EV production combines decisive assets for clinical translation of EV-based regenerative medicine : a GMP-compliant setup, high density cell culture, high yield release of EVs per cell, high purity EVs.

OF11.04

Prolongation of allograft survival via donor MHC chimerism induced by extracellular vesicles

Bruno Adonai Gonzalez Nolasco[a], Mengchuan Wang[a], William Orent[a], Aurore Prunevieille[a], Jane O[a], Kaitlan Ahrens[a], Joren C Madsen[b] and Gilles Benichou[a]

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Introduction: Achieving robust and durable host immune tolerance of allogeneic transplants is the ultimate goal in clinical transplantation. Mixed chimerism induced via donor bone marrow transplantation and host non-myeloablative conditioning has reliably achieved tolerance of allogeneic organ transplants in mice and humans. Tolerance in this model is believed to rely essentially on the presentation of donor MHC molecules in the host's thymus. In this study, we investigated whether donor MHC chimerism could be achieved via donor extracellular vesicles (EVs) injections and subsequent cross-dressing of recipient cells in the host's thymus.
Methods: Conditioned SJL (CD45.1+, H2-Ks+) recipient mice received a single IV dose of purified bone marrow derived exosome-enriched EVs (BM-EVs) isolated from C57BL/6 (CD45.2+, H2-Kb+) donors through sequential centrifugation or using a commercially available exosome isolation kit. Nanoparticle tracking showed vesicles of approximately 100nm in size in the BM-EVs preparation and Western Blot showed the presence of MHCI. Image flow cytometry was used to detect the presence of cross-dressed cells from day 10 through 100 after exosome injection. For NHP studies, MHC class I H38+ BM-EVs were injected into a H38- conditioned cynomolgus macaque prior to a combined heart and kidney transplant. PBMCs, thymus, spleen and mesenteric lymph nodes were collected for image flow cytometry.
Results: Intravenous injection of BM-EVs into conditioned mice resulted in the presentation of donor MHC and CD45.1 molecules by host's thymic and splenic cells. Similarly, H38+cross-dressed cells were detected at D33 after exosome injection in all of the NHP recipient tissues collected. In mice, donor but not syngeneic or third-party BM-EVs significantly prolonged skin allograft survival (median survival = 17 VS 11 days, $p < 0.001$).
Summary/Conclusion: These results show that delivery of donor-derived extracellular vesicles can induce donor MHC chimerism via cross-dressing of recipient APCs with allogeneic MHC molecules in the host's thymus. This suggests that donor EVs could be used in place of bone marrow cells to induce chimerism and allograft survival with minimal conditioning and no risk of graft versus host disease (GVHD).
Funding: NIH R01DK115618.

OF11.05

Proteomic and transcriptomic characterization of exosomes-mimetic nanovesicles reveals their relevance as a therapeutic delivery system
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Introduction: The potential of utilizing exosomes (endosomal derived vesicles) as a therapeutic delivery system of biological and chemical drugs are an active area of clinical phase investigation. However, the field is currently facing challenges such as the insufficient release of exosomes, their heterogeneity and reproducibility of isolation. These issues may be overcome through the development of artificial extracellular vesicles (EVs). Cell-derived mimetic nanovesicles (M-NVs) can be generated from numerous cell lines with advantages such as, reproducibility, large scale production, uniformity, cost effectiveness and a simple purification method. Although several studies have shown that M-NVs have similar morphology, size and therapeutic potential to exosomes, comprehensive characterization and to what extent these artificial EV components mimics exosomes remain elusive.
Methods: In this study, M-NVs generated by subjecting cells to the extrusion, were comprehensively characterized and compared to the exosomes by proteomic and transcriptomic analysis.
Results: We analysed the proteome between M-NVs and exosomes to provide key insights into key membrane surface features of exosomes for cargo sorting and therapeutics delivery are preserved in M-NVs (158 proteins). Furthermore, our study highlighted differences in protein post-translational modifications among M-NVs, as distinct from exosomes, using a non-targeted informatic approach, specifically showing phosphorylation, ubiquitination, and thiophosphorylation as enriched protein modifications in M-NVs. Small RNA analysis reveals that unlike exosomes, the RNA cargo of M-NVs is similar to that of the parental cells. In addition, we found that M-NVs could be useful for packaging proteins or RNA which are globally enriched in cells. Indeed, this may overcome the challenges involved in selective packaging of therapeutic proteins or RNAs into exosomes.

Summary/Conclusion: In summary, results from this study provides key insights into omics of M-NVs cargo in comparison to exosomes and ultimately its potential as therapeutic delivery system.

Funding: Grants from the Australian Research Council, Lundbeck Foundation and the Danish National Mass Spectrometry Platform for Functional Proteomics.

OF11.06

Exosomes from periodontal ligament-derived cells promote cutaneous wound healing and topical application is superior to local injection

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Introduction: Periodontal ligament-derived mesenchymal stromal cells (PDL-MSCs) represent an attractive source of cells for regenerative medicine due to four reasons; 1) similarly to other MSCs, they exhibit pro-regenerative properties, 2) accessibility is great due to abundance of extracted teeth, 3) they can easily be expanded and 4) they can be used in an allogeneic fashion. We are currently investigating the use of sheets produced from these cells to regenerate periodontal tissue. In this study we aimed to investigate the potential of exosomes from human PDL-MSCs to stimulate wound healing.

Methods: We used ultrafiltration and size-exclusion chromatography to isolate vesicles from serum-free conditioned media. BCA-assay and nanoparticle tracking analysis (NTA) was used to determine yield. We performed Western Blot for positive and negative extracellular vesicle-markers, and transmission electron microscopy was used to evaluate morphology. We then performed wound healing assay in immunocompetent rats. Each rat received two full-thickness wounds, treated with either topical application or perilesional injections, and PBS was used in control rats. The animal weights were measured and wounds were photographed every other day. The animals were sacrificed on day 7 and the tissue was collected for histopathological analysis.

Results: Exosome yield was on average 0.83 μg proteins per million cells per 24 h. The exosomes had a mean size of 130 nm, showed positivity for CD9 and Flotillin-1 and negativity for GRP94 and had a spherical morphology. The exosomes were applied to wounds and rats receiving exosomes gained significantly more weight than controls. Topical application proved to be superior to injections based on macroscopic wound evaluation and histopathology.

Summary/Conclusion: PDL-MSC-derived exosomes stimulate wound healing in a xenogeneic setting and topical application is superior to local injections.

Funding: This work was supported by The Swedish Society of Medicine, Erik och Edith Fernströms stiftelse för medicinsk forskning, Misao-Yanagihara-Grant for regenerative medicine research and JSPS KAKENHI Grant Number JP18H02985.

Symposium Session 12: Protein Biomarkers in Human Disease
Chairs: Malene Møller Jørgensen; Koji Ueda
Location: Level B1, Lecture Room

08:30–10:00

OF12.01

Biomarkers of peritoneal membrane alteration in dialysis efflux-extracellular vesicles: a longitudinal study in patients under peritoneal dialysis treatment

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Introduction: Peritoneal dialysis (PD) is considered the best renal replacement therapy for patients waiting for a kidney transplant. Many patients eventually suffer ultrafiltration failure of the peritoneal membrane (PM), leading to severe clinical complications and the urgent need to change the dialysis technique. Currently, PM functionality is monitored by the peritoneal equilibration test (PET), a tedious technique that only shows changes when the PM damage is advanced. We hypothesized that peritoneal dialysis efflux (PDE)-extracellular vesicles (EV) may contain biomarkers of PM state. In a previous study (Carreras-Planella, et *al*., PLoS One 2017), we showed for the first time that PDE-EVs could be isolated and their protein content showed differences between newly enrolled and long term PD patients. Here, we present the results of a longitudinal study in a new cohort of PD patients.
Methods: PDE was collected from 12 PD patients every 6 months (coincident with PET controls) up to 24 months follow up. PDE-EV were isolated by size-exclusion chromatography and characterized by expression of classical tetraspanin EV markers. EV proteome was analysed by Mass Spectrometry (LC-MS/MS).
Results: In accordance with our previous study, PDE-EV proteome showed reduced expression of several proteins at longer timer points (>12 months) of treatment. In addition, statistical analysis revealed confidently identified proteins – potentially involved in fibrotic processes – that are significantly deregulated in patients showing alterations in PET monitoring at 12 months of treatment.
Summary/Conclusion: Our results confirm the potential of analysing PDE-EV as biomarkers of PM alteration allowing improved monitoring of PD patients compared to PET.
Funding: The IGTP is member of the CERCA network of institutes. LCP is sponsored by the FPU scholarship (FPU17/01444) from the Ministerio de Ciencia, Innovación y Universidades of the Spanish Government. MF is sponsored by the PERIS contract SLT002/16/00069, from the Generalitat de Catalunya. F.E.B. is a researcher from Fundació Institut de Recerca en Ciències de la Salut Germans Trias i Pujol supported by the Health Department of the Catalan Government (Generalitat de Catalunya).

OF12.02

Proteomics of urine-derived extracellular vesicles to identify biomarkers of prostate cancer risk groups

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Introduction: Prostate cancer (PCa) is the most common cancer in men and can be detected early through screening of asymptomatic men. Most prostate cancers are indolent at time of diagnosis and current prognostic protocols do not accurately predict disease aggression and clinical outcome, which limits optimal patient management. For example, high serum prostate-specific antigen (PSA) levels could be indicative of metastatic cancer or benign prostatic conditions, while needle biopsies are invasive and can undersample the prostate, resulting in uncertainty of cancer grading. We hypothesize that small extracellular vesicles (sEVs) isolated from post-digital rectal exam urine (pDRE-urine) contain protein biomarkers will allow for non-invasive PCa risk stratification.

Methods: We have performed deep proteomics analysis on pDRE-urine-derived sEVs from 105 treatment-naïve, richly annotated patients (age, T-stage, Gleason score, PSA, etc.). sEVs were isolated by differential ultracentrifugation and processed for proteomics (LC-MS). Size and morphology of sEVs were verified by nanoparticle tracking analysis and TEM.

Results: We detected 3,688 proteins in sEVs, 80% of which are shared with the prostate cancer tissue proteome (unpublished). sEV proteins were enriched in cytoplasmic and membrane proteins and depleted in nuclear proteins. Interestingly, sEVs were also enriched for prostate-specific proteins compared to the proteome of urine that was analysed in parallel, suggesting enrichment for low-abundance tissue-originating protein cargo in sEVs. Samples clustered into three groups based on global protein expression, suggesting that there may be subtypes of sEVs within pDRE-urine.

Summary/Conclusion: We are currently applying machine learning approaches to identify biomarkers that could supplement current diagnostic tests and improve stratification of patient risk groups. In the future, we will confirm differential protein expression by targeted proteomics assays using an active surveillance cohort and perform parallel profiling of sEV RNA cargo. Ethics approval at University Health Network.

Funding: National Cancer Institute-Early Detection Research Network.

OF12.03

Extracellular vesicle biomarkers predict Alzheimer's disease in the baltimore longitudinal study of ageing

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Introduction: It was recently reported that plasma neuronal-enriched extracellular vesicles (EVs) of Alzheimer's disease (AD) patients exhibit elevated levels of phosphorylated tau, Aβ42, and phosphorylated insulin receptor substrate-1 (IRS1). To validate them as AD predictors, we interrogated preclinical samples from Baltimore Longitudinal Study of Ageing participants.

Methods: We blindly analysed 931 longitudinal plasma samples from 138 cognitively normal participants who eventually developed AD (cases) and 233 age and sex-matched Controls who remained cognitively normal. The earliest samples preceded AD symptom onset by a median of 4.1 years. We precipitated total particles using Exoquick and then immunoprecipitated neuronal-enriched EVs using antibody against neuronal cell adhesion molecule L1CAM. We lysed isolated EVs and quantified proteins by immunoassays. We adjusted values for EV concentration and diameter to normalize for EV yield. We compared cross-sectional and longitudinal trajectories of EV biomarkers between future AD and Control participants and performed stepwise logistic regression with internal cross-validation and receiver operating characteristic analysis to assess the ability of EV biomarkers to discriminate future AD cases from Controls.

Results: Future AD cases had cross-sectionally and longitudinally higher p181-Tau, p231-Tau, pSer312-IRS1, pY-IRS1 and EV diameter than Controls but similar Aβ42, total Tau, TSG101 and EV concentration. A model optimally combining longitudinal data for multiple biomarkers achieved 90.2% sensitivity (95% confidence interval [CI], 81.2–95.4%), 83% specificity (95% CI, 76–88%) and 91.6% area under-curve (95% CI, 87.9–95.4%) for predicting AD. Preclinical levels of several EV biomarkers were associated with cognitive performance.

Summary/Conclusion: We validated several neuronal-enriched EV biomarker candidates and further demonstrated that their preclinical longitudinal trajectories predict AD diagnosis with high sensitivity. These findings motivate further development of EV biomarkers towards a clinical blood test for AD.

Funding: This research was supported entirely by the Intramural research Program of the NIH, National institute on Aging

OF12.04

CD315 (PTGFRN) – a new biomarker for tumour-derived extracellular vesicles

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Introduction: Extracellular vesicles (EVs) represent important mediators of cell-cell communication and are secreted by many types of cells, including tumour cells, into the extracellular milieu. Tumour-derived EVs hold a lot of promise for non-invasive diagnostic tests, also known as liquid biopsy, because they are

present in all kind of biological fluids and carry a large variety of proteomic and genetic information. There is now an ever-growing need for new specific biomarkers, which allow for the isolation of distinct EV subclasses in order to improve EV-based diagnostics. We show for the first time that CD315 (also known as PTGFRN, EWI-F or CD9P-1) may represent a new potential biomarker for tumour-derived EVs.

Methods: The expression of CD315 was studied in cell lines, primary tumour samples and corresponding EVs. CRISPR/Cas9 CD315 knockout cells were used to investigate the impact of CD315 on cell proliferation and EV secretion. Furthermore, we generated a CD315-specific monoclonal antibody to elucidate the diagnostic potential of CD315+EVs in blood samples of cancer patients.

Results: We demonstrated that CD315 is highly expressed on a large variety of tumour cells and is present on the surface of tumour-derived EVs. *In vitro* knockout of CD315 hampered proliferation and migration of tumour cells and affected cellular EV production. Moreover, our CD315-specific antibody was successfully used to capture and isolate CD315 +EVs by immunoaffinity.

Summary/Conclusion: We identified CD315 as a promising new biomarker with diagnostic potential. Although its specific function still remains to be elucidated, we were the first to show that CD315 is highly abundant in tumour-derived EVs. Additionally, we generated a CD315-specific antibody as a valuable tool for immunoisolation of distinct EV subclasses.

OF12.05

Analysis of urinary extracellular vesicles auto fluorescence in imaging flow cytometry and spectral flow cytometry.

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Introduction: Urinary extracellular vesicles (uEVs) provide a source of valuable biomarkers for kidney and urogenital diseases. Analysis of uEVs in imaging flow cytometry is challenging for its intrinsic natural auto fluorescence emission across the whole electromagnetic spectrum. To date it is not known what the rate of the autofluorescence interference is with respect to the detection of specific marker uEVs markers

Methods: First morning void urine and citrate blood from the same donor were centrifuged at 4,600 g for 30 and 15 min, respectively. The supernatant was centrifuge at 20,000g to collect urinary (uEVs) and plasma (pEVs) which were stained with the same commercial clone antibody (3D3) anti podocalyxin (PODXL) conjugated with 3 different fluorescent dyes: Alexa Fluor® 405 (AF405), Alexa Fluor® 488 (AF488) and Alexa Fluor® 647 (AF647). Stained EVs were acquired with both imaging flow cytometry and spectral flow cytometry. Gate strategy was based on the low scatter of the unstained uEVs and the negative control was the fluorescent probe alone in buffer.

Results: Acquisition of uEVs alone showed auto-fluorescence emission in channel 2 (λex 488 nm; λem 480–560 nm) camera 1 and channel 11 (λex 658 nm; λem 660–740 nm) but not channel 7 (λex 405 nm; λem 420–505 nm) for camera 2 for the imaging flow cytometry meanwhile the spectral flow cytometry revealed a spectral fingerprint spanning from the violet to the red emission. Autofluorescence was detected for uEVs but not pEVs. Podocalyxin-AF405 conjugated stained both uEVs and pEVs with a double staining for the auto-fluorescence and PODXL on the same uEV. While PODXL-AF488 and AF647 stained pEVs both the antibody conjugated failed to detect the uEVs as per PODXL-AF405. Same results were obtained for both flow cytometry instruments.

Summary/Conclusion: While imaging flow cytometry represent a major advancement in the identification of uEVs, our results showed an unexpected additional complication of the analysis originated from the auto-fluorescence of the uEVs fraction. In fact, The auto-fluorescence quenched the emission of PODXL-AF488 and AF647 but not AF405. uEVs auto-fluorescence needs to be taken into account especially when simultaneous co-detection of uEVs markers of podocyte origin is planned with particular emphasis on the critical selection of the antibody conjugated fluorescent dye.

OF12.06

Serum vs. plasma: a comparative study in EV composition

Razieh Dalir Fardouei[a], Rossella Crescitelli[b], Aleksander Cvjetkovic[a], Jan Lötvall[c] and Cecilia Lasser[d]

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Introduction: The ability to isolate extracellular vesicles (EVs) from blood is paramount in the development of EVs as disease biomarkers. However, this is complicated by the profuse presence of plasma proteins and lipoprotein particles, making blood one of most difficult body fluids to isolate EVs from. We have previously developed a method to isolate EVs from blood with minimal contamination of lipoprotein particles (Karimi et al 2018). The aim of this study was to compare the amount of EVs and their protein cargo isolated from plasma and serum.

Methods: Blood was collected from healthy subjects, from which plasma and serum were isolated. EVs were isolated using a combination of density cushion and size exclusion chromatography (SEC). Purity and yield of EVs were determined by nanoparticle tracking analysis (NTA), Western blot, electron microscopy (EM), and mass spectrometry (LC-MS/MS). Additionally, Cy7-labelled cell line-derived EVs were spiked in to blood prior to isolation of plasma and serum to compare the recovery.

Results: As determined by NTA and protein measurement more EVs could be isolated from plasma. This result was supported by experiments were labelled EVs were spiked in to blood, which demonstrated that less labelled EVs could be retrieved from serum compared to plasma. Enough plasma EVs could be isolated for proteomic analysis from 12 ml blood, which was not possible for serum-derived EVs from the same amount of blood. When larger amount of serum and plasma was used as starting material 1789 proteins could be identified in plasma-derived EVs, while only 628 proteins could be identified in serum-derived EVs. Both proteomes were strongly associated with the GO term "Extracellular exosome", while the serum derived EVs were more associated with "Complement activation".

Summary/Conclusion: This study shows that a larger amount of EVs could be isolated from plasma compared to serum. We currently don't have the explanation why this is so, however it might be due to the fact that EVs get trapped in the clot during serum formation. Future studies are needed to answer how this affects the use of blood-derived EVs as biomarkers from serum and plasma.

Symposium Session 13: Stem Cell Derived EVs
Chairs: Qingling Fu; Tatiana Lopatina
Location: Level 3, Hall B

08:30–10:00

OF13.01

Extracellular vesicles confer DNA damage on residual long-term HSC in the AML niche

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Introduction: Acute Myeloid Leukaemia (AML) is a hematopoietic cancer that arises from mutations in hematopoietic stem cells (HSC). Genomewide sequencing has revealed that patients harbour multiple leukaemic clones operating in dynamic succession. Molecular abnormalities have also been uncovered in phenotypically normal residual HSC from AML patients. Independently, several groups showed enforced quiescence in residual long-term (LT-) HSC in the AML microenvironment. Neither observation is fully understood.

Methods: Our previous studies in AML xenografts showed that extracellular vesicles (EV) contribute to the erosion of hematopoietic progenitor function. Here we hypothesized that AML EV may similarly shape fate and function of residual HSC in the AML niche. We used a combination of in vivo, ex vivo and in vitro approaches and utilizing both AML cell lines and primary AML patient cells.

Results: We confirmed the relative enrichment of residual HSC in the BM due to gains in quiescence even at low leukaemic burden, or following AML EV injections. We also observed *in vivo* AML-EV trafficking to LT-HSC associated with p53 hyperphosphorylation, but not apoptosis or senescence. Next, a proteomic screen of EV-exposed HSPC identified the systematic suppression of ribosome biogenesis as the most highly enriched Gene Ontology category. The mTOR pathway governs ribosome biogenesis and protein synthesis, and we went on to show that AML-EV trafficking of micro RNA-1246 targets Raptor, a pathway component. Translational suppression of Raptor In turn caused ribosomal protein S6 hypo-phosphorylation and suppressed protein synthesis. Quiescent HSC are known to rely on error prone mechanisms of DNA repair, and we demonstrate that residual HSC accrue DNA damage, gain replating competency and show increased in vivo repopulation.

Summary/Conclusion: Altogether, our studies suggest that EV miRNA dysregulate proteostasis and confer HSC quiescence in the AML BM. We uncover evidence of long-lasting DNA damage in residual LT-HSC via AML EV.

Funding: Institutional,; Hyundai Hope on Wheels Foundation.

OF13.02

Extracellular vesicles contribute to the development of ionizing radiation-induced late bone marrow pathologies

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Introduction: Bone marrow (BM) is a particularly radiosensitive organ; haematological malignancies, myelodysplastic syndrome and chronic bone marrow insufficiency are considered long-term consequences of bone marrow irradiation. Ionizing radiation (IR) damages the stem and progenitor cells and alters signalling between the stem cell compartment and the BM stroma. The major objective of our work was to investigate extracellular vesicles (EVs) mediated IR effects in the BM and stroma at low and high irradiation doses and to study possible underlying mechanisms using an *in vivo* murine model.

Methods: C57Bl/6 mice were irradiated with 0.1 Gy or 2 Gy and EVs isolated from the BM supernatant were injected systemically into naive animals. EV-mediated phenotypical changes were determined by flow cytometry in the stem and progenitor cell compartment and in the BM stroma. Apoptosis in various cellular subpopulations was measured by Tunnel assay, DNA damage by immunostaining using the γH2AX assay, senescence by β-gal staining. Oxidative damage was evaluated in the BM cells by measuring protein oxidation and lipid peroxidation and systemically by determining the level of 8-hydroxy-2' -deoxyguanosine in the urine.

Results: Treatment of naïve mice with BM-derived EVs from irradiated animals induced apoptosis in certain cellular subpopulations, led to local and systemic oxidative damage, decreased the number of haematopoietic and mesenchymal stem cells and of lymphoid progenitors, changed the ratio between the long term and short term stem cells, increased systemic release of immature progenitors into the circulation. Stroma was less affected; endothelial cells were the most sensitive.
Summary/Conclusion: BM-derived EVs mediated IR-induced damage in the bone marrow and stroma, which raise the role of BM-derived EVs in the development of IR-induced late BM pathologies.
Funding: Euratom research and training programme 2014–2018 under grant agreement No 662287 (CONCERT)

OF13.03

Myeloid derived extracellular vesicular WNT induces rectal stem cell regeneration
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Introduction: Rectal epithelial injury is the major limiting factor for pelvic radiotherapy. Activation of regenerative response of rectal stem cells (RSCs) is critical to mitigate radiation injury. Wnt β catenin signalling plays a critical role in homeostasis and regeneration of intestinal stem cell (ISC). Both epithelium and stroma are the major source of WNT ligands. Intestinal stroma consists of several cell types including mesenchymal cells and myeloid/macrophages (Mφ). Genetic or pharmacological inhibition of WNT release from mesenchymal stromal cells did not affect the ISC homeostasis or regeneration. In the present study we have examined the effect of Mφ derived extracellular vesicle (EV) packaged WNT in homeostasis and repair of RSCs.
Methods: Csf1r.iCre;Porcnfl/fl mice deficient in Mφ derived WNT due to Mφ-restricted ablation of Porcupine, a gene essential for WNT synthesis were used to determine effect of Mφ derived in EV-WNT in RSC homeostasis and regeneration. Mice were exposed to lethal dose of pelvic irradiation (PIR) (18Gy) to deplete RSCs and therefore evaluate the regenerative response following treatment with Mφ derived EV packaged WNT. Effect of Mφ-EV WNT on RSCs were also examined in ex-vivo rectal organoid system developed from Lgr5/GFP-IRES-Cre-ERT2 knock-in for visualization and quantification of Lgr5+ve RSCs.
Results: Histopathological analysis of Csf1r.iCre; Porcnfl/fl mice rectum demonstrated no differences in epithelial morphology compared to wild type mice. However, exposure to PIR which depletes all RSCs demonstrated higher radio-sensitivity and significant damage in rectum epithelium in Csf1r.iCre;Porcnfl/fl mice compared to wild type mice. EV purified from Mφ conditioned medium demonstrated presence of functionally active WNT ligands and improve regenerative capacity of RSCs in both human and mice rectal organoid model ex-vivo. Treatment with Mφ conditioned medium containing EV promote regenerative capacity of Lgr5+ ve RSCs in Lgr5/GFP-IRES-Cre-ERT2 knock-in mice exposed to PIR. However, treatment with EV depleted condition medium failed to rescue RSCs against irradiation.
Summary/Conclusion: Homeostasis of rectal epithelium is not dependent on Mφ derived EV packaged WNT. However, Mφ derived EV packaged WNT is critical for regenerative response of RSCs against injury.

OF13.04

Glycome analysis of extracellular vesicles derived from stem cells using lectin microarray
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Introduction: In addition to proteins, nucleic acids and lipids, extracellular vesicles (EVs) are also composed of glycans. EV glycome may provide vital clues for a better understanding the biogenesis, release and transfer of vesicles. However, little is known regarding glycans on EVs. Do glycans on EVs change depending on cell types and cellular conditions? More specifically, do stem cell-derived EVs carry stem cell glycan markers? Such basic questions remain unclear.
Methods: Here, we performed glycome analysis of EVs derived from stem cells including human induced pluripotent stem cells (hiPSCs) and human messenchymal stem cells (hMSCs) using high-density lectin microarray and flow cytometry.
Results: Detailed analysis of the results obtained by lectin microarray and flow cytometry revealed that hiPSC-derived EVs carry characteristic features of cell surface glycans. rBC2LCN, a specific lectin for hPSCs, bound to hiPSC-derived EVs, but not to non-hiPSC-derived EVs. One of the glycoprotein ligands of rBC2LCN on EVs was identified as podocalyxin, which is a cell surface glycoprotein ligand of rBC2LCN. Other hiPSC surface glycan markers were

also detected on the surface of EVs. Finally, we developed a sandwich assay to specifically detect hiPSC-derived EVs using rBC2LCN and Tim4, which binds to phosphatidylserine (PS). rBC2LCN is useful for the specific detection of hiPSC-derived EVs.

Summary/Conclusion: The EV glycome reflects its cellular origin, which could be a novel target for the development of the quality control system of stem cells used for regenerative medicine.

Funding: JST CREST

OF13.05

Exosomes derived from human MSC mediate monocyte mobilization to orchestrate neovascularization in radiation-induced skin injury

Alexandre Ribault[a], Bruno Lhomme[a], Celine Loinard[a], Marc Benderitter[a], Stephane Flamant[a], Ruenn Chai Lai[b], Sai Kiang Lim[c] and Radia Tamarat[a]

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Introduction: Emerging evidences indicate that extracellular membrane vesicles, such as exosomes, could recapitulate the therapeutic effects of huMSC. Of note, exosomes displayed marked pro-angiogenic activity, however a better understanding of their underlying mechanisms of action remained to be defined. This study aims to investigate the mechanisms governing the pro-angiogenic effects of huMSC derived exosomes (huMSC-Exo) in a mouse model of radiation-induced musculo-cutaneous injury.

Methods: Mice lower limb was exposed to 80Gy X-ray irradiation to induce radiation injury. After 14 days, mice received an intramuscular injection of 106 human MSCs, 400 μg MSC-EXO, or PBS. Angiogenesis was estimated by skin perfusion (laser Doppler imaging), immunohistochemistry (CD 31 endothelial marker) and microangiography (barium sulphate). Mice were sacrificed at several time points, and tissues of both irradiated and contralateral limbs were harvested for histological and biochemical analyses. Bone marrow, spleen and blood were collected for analysis of inflammatory cells and circulating factors. In vitro assays were used to validate the pro angiogenic effet of HuMSC- exo.

Results: The huMSC-Exo stimulated vascular growth as revealed by the increase in cutaneous blood perfusion, capillary density and angiographic score with stimulation of pro-angiogenic factor levels such as VEGF-A and eNOS. In vitro, huMSC-Exo fostered endothelial cells and fibroblast migration in a PI3K/AKT and TGF-β/SMAD2 dependent pathways. Finally, huMSC-Exo triggered the mobilization of both Ly6Chi and Ly6Clo monocytes from the spleen and the bone marrow and their recruitment into the irradiated muscle. Additionally, monocyte and macrophage depletion through clodronate treatment completely abrogated the pro angiogenic effect of huMSC-Exo.

Summary/Conclusion: This study demonstrates, for the first time, that huMSC derived exosomes enhance the angiogenic process in the radiation-induced ischemic tissue by stimulating the mobilization and recruitment of innate cells to the lesion and nurturing neovascularization. These results highlight the concept that huMSC-Exo administration represents a suitable innovative approach for therapeutic angiogenesis in irradiated tissue.

OF13.06

hucMSCs derived exosomes enhance lymphangiogenesis in experimental lymphedema via exosomal transfer of Ang-2 and Tie2

Ting Zhao and Yongmin Yan

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Introduction: Exosomes are small biological membrane vesicles secreted by cells, including MSCs. Here, we evaluated lymphangiogenic potential and key exosomal prolymphangiogenic factors of human umbilical cord MSC-derived exosomes (hucMSC-Ex) to providing a mechanistic basis for optimizing future hucMSC-Ex-based lymphedema therapies.

Methods: hucMSC-Ex were extracted from condition medium of hucMSCs. Using a murine lymphedema model, we evaluated oedema at various time points post hucMSC-Ex injection. HE stain and Immunohistochemical stain were applied to analyse the lymphaniogenesis. In vitro, human dermal lymphatic endothelial cells (HDLECs) were treated with hucMSC-Ex, and cell proliferation, migration and tube formation were assayed using cell counting Kit-8 (CCK-8), transwell chamber inserts, and matrigel-based tube formation assays, respectively. Western blot and immunofluorescence stain were performed to test the expression level of proteins which were associated with lymphaniogenesis after co-cultured with hucMSC-Ex in HDLECs.

Results: Mice treated with hucMSC-Ex showed significantly decreased oedema formation and restored drainage of intradermally injected methylene blue after 6 weekly injections. HE stain showed subcutaneous oedema of tail faded obviously after hucMSC-Ex injection. Immunohistochemical analysis revealed that mice tails receiving hucMSC-Ex injections had enhanced lymphangiogenesis compared to the PBS-treated groups as determined by staining of lymphatic marker LYVE-1. The proliferation, migration, and tube

formation of HDLECs were significantly increased by hucMSC-Ex. Also, the expression level of Ang-2, Lyve1, Prox1, VEGFR3, p-Akt in HDLECs was up-regulated both in western blot and Immunofluorescence stain. Mechanically, hucMSC-Ex derived Ang-2 and Tie2 proteins were transferred to HDLECs. Ang-2 controlled the proliferation, migration and tube formation of HDLECs. And hucMSC-Ex delivered Ang-2 and Tie2 activated the expression of lymphangiogenic factors.

Summary/Conclusion: Ang-2 and Tie2 are essential for hucMSC-Ex effects on lymphangiogenesis in vitro and in vivo.

Funding: Zhenjiang Key Laboratory of Exosomes Foundation and Transformation Application High-tech Research,china: (ss2018003);National Natural Science Foundation of China: (81670549)

Symposium Session 14: Parasite and Bacterial EVs
Chairs: Yong Song Gho; Mariko Ikuo
Location: Level B1, Hall A

08:30–10:00

OF14.01

Macrophage-derived exosomes encapsulate Salmonella antigens and stimulate the activation of Type 1 T-helper cells in vivo

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Introduction: *Salmonella* enterica *serovar Typhimurium* is a Gram-negative, intracellular bacterium which invades macrophages and leads to the production of pro-inflammatory exosomes. *S. Typhimurium* is the causative agent of salmonellosis affecting 1.2 million people annually in the USA. There are no FDA approved vaccines against non-typhoidal Salmonella infections for human thus showing a significant limitation in current prevention methods. Exosomes are a subclass of extracellular vesicles characterized by their size, morphology and biogenesis. The cargo, including protein, nucleic acids and metabolites, carried by exosomes vary depending on the physiological conditions present and the origin of the cell. We hypothesize that during *Salmonella* infections, exosomes transport *Salmonella* antigen to alert neighbouring cells which can lead to the stimulation of naïve T-lymphocytes.

Methods: We focus on the release of exosomes by *S. Typhimurium*-infected macrophages and their function in stimulating an adaptive immune response in vivo. To determine if exosomes have any effect on the adaptive immune response, mice were given doses of exosomes derived from *S. Typhimurium* infected macrophage. Fluorescent activated cell sorting was used to monitor T- lymphocyte response.

Results: Exosomes stimulate a distinct cytokine secretion pattern among CD4+T lymphocytes *in vivo*. The cytokines milieu, including IFN-, TNF- and IL-2, expression by T-lymphocytes suggest that the CD4 T-lymphocytes differentiated in to Type 1 T-helper set producing pro-inflammatory cytokines. Additionally, mouse serum was taken to analyse for antibody production against Salmonella in which we observe exosomes derived from Salmonella infected cells provide a similar antibody production to the live vaccine. Based on our -omics study, we identify Salmonella antigens and other pro-inflammatory molecules in exosomes isolated from *Salmonella* infected-macrophages from 24 and 48 h infections. Hence, the cargo plays a critical role in intercellular communication in response to infection as naïve macrophages treated with these exosomes result in M1 polarization.

Summary/Conclusion: Our data support the hypothesis that exosomes isolated from Salmonella infected macrophages carry Salmonella antigens as a cargo and stimulates the activation of Type 1 effector T lymphocytes.

OF14.02

Extracellular vesicles from *Leishmania donovani* infected macrophages contain infection-specific cargo that contribute to lesion development

Anna E. Gioseffi and Peter Kima

University of Florida, Gainesville, USA

Introduction: Extracellular vesicles (EVs) have emerged as important mediators of cell-to-cell communication and have been shown to contribute to the pathogenesis of infectious microorganisms. *Leishmania* is an intracellular eukaryotic parasite and causative agent of leishmaniasis. This work aims to evaluate EVs in the context of *Leishmania donovani* infection.

Methods: To better understand the properties and function of EVs produced by *L. donovani* infected RAW264.7 macrophages (iEVs), we used a series of approaches, including comparative proteomics of iEVs or EVs derived from uninfected RAW 264.7 macrophages, pathway analysis to infer activity, and functional assays such as in vitro migration assays and flow cytometry to evaluate endothelial cell activation after EV treatment.

Results: We obtained a profile of host and parasite proteins in iEVs, EVs from uninfected macrophages, and EVs from macrophages infected with Centrin knockout (CenLd) parasites. CenLd parasites are unable to mature into the amastigote form within macrophages. In addition to host derived molecules previously identified by others in exosome

preparations, we identified host and parasite derived molecules, such as parasite PI3K, vasohibin, and serine/threonine protein phosphatase, and mouse histone 2B, annexin A3, and galectin-3 within iEVs. Our results showed that EVs from macrophages infected with CenLd parasites have a molecular composition that is qualitatively different from iEVs released by macrophages infected with wild type parasites. Pathway analysis of the host-derived proteins in iEVs suggested their involvement in cell migration and neovascularization. In vitro cell migration assays validated the observation that intact iEVs enhance cell migration, which is in contrast to EVs from Cen-/-Ld infected macrophage or disrupted iEVs that do not induce cell migration. In addition, flow cytometry analysis of endothelial cells treated with iEVs suggested that EVs from infected macrophages activate endothelial cells. The latter observations were surrogate indicators of pathogenic neovascularization.

Summary/Conclusion: Taken together, we present a comprehensive molecular profile of the molecules released in EVs from *L. donovani*-infected macrophages which includes infection-specific molecules that contribute to lesion development.

Funding: NIH.

OF14.03

The role of extracellular vesicles in neurophysiological changes induced by chronic *Toxoplasma gondii* infection

Ellie Tedford[a] and Glenn McConkey[b]

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Introduction: During chronic *Toxoplasma gondii* infection, parasites become encysted in neurons in the host brain. Infection is associated with a plethora of behavioural and neurophysiological changes despite only a small number of neurons infected. Perturbations in dopamine metabolism have been observed with infection. We have previously described parasite induced down-regulation of dopamine β-hydroxylase. In this study, the role of extracellular vesicles during chronic *T. gondii* infection was investigated.

Methods: Primarily transwell culturing and extracellular vesicle purification via ultracentrifugation were used during this study. Extracellular vesicles were collected from chronically infected human and rat catecholaminergic cells. Purification via a 12 step sucrose gradient was performed prior to conditioning *in vitro* and *in vivo*.

Results: Altered DBH mRNA expression was identified during *T. gondii* infection in rat catecholaminergic and human neuronal cells. This down-regulation of DBH was identified in de novo RNA, suggesting that regulation occurs at the transcriptional level. Using MSRE-qPCR, hypermethylation in the 5‘ upstream region of the DBH promoter was identified in infected rat catecholaminergic cells and human neuronal cells. Surprisingly, DBH mRNA down-regulation and methylation in the 5’ promoter were globally altered *in vivo*, despite the fact that only a small number of cysts can be identified in the host brain. DBH silencing was observed in cells only exposed to infected cells. Extracellular vesicles purified from infected rat catecholaminergic cells induced transcriptional silencing of DBH. This effect could also be generated in vivo when rats received intracererbral injections with purified extracellular vesicles from infected cells. This represents a new perspective of the host-pathogen interaction.

Summary/Conclusion: Through this mechanism *T. gondii* may be able to induce many of neurophysiological changes associated with chronic infection. In addition, *T. gondii* is a unique model to study mammalian neurological function, by examining the influence the parasite is able to exert over cell-cell communication, we may be able to further understand the mechanisms governing CNS function and dysfunction.

Funding: The University of Leeds.

OF14.04

Bacterial membrane vesicles as vaccines in aquaculture

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Introduction: Infections by two Gram-negative intracellular bacterial pathogens *Piscirickettsia salmonis* and *Francisella noatunensis*, are causing major problems in aquaculture world-wide. *F. noatunensis* sp hampers the development of fish farming based on cod in and is deleterious to tilapia. *P. salmonis* infections have been devastating for salmon aquaculture. As of today no effective treatments are available against the diseases. Both *P. salmonis* and *F. noatunensis* secrete membrane vesicles (MV). Bacterial MV has been reported as potential vaccine candidates for a range of host including humans, mice and fish against infection caused by intracellular pathogenic bacteria as they induce both a humoral and cellular immunity.

Methods: We have isolated MVs from both Francisella and Piscirickettsia by the ultracentrifugation Method. The MVs were characterized by their size distribution, by transmission electron microscopy (TEM) and proteomics. Their toxicity were tested by injecting MVs into both our zebrafish vaccine and challenge model as well as in cod, tilapia and salmon. A vaccine trail was performed first in our zebrafish model, and then in cod, tilapia and salmon.
Results: The MV size analysis showed that the MVs size distribution ranged from 20–250 nm in size with most ranging from 70–100 nm. Both single and double membrane MV were found in the population as investigated by TEM. Further, immune-gold labelling revealed the presence of DNA in both populations. Proteomics analysis revealed that the MV content varied between bacterial strains. Immunization with MV gave protection against disease caused by both *P. salmonis* and *F. noatunensis* in our zebrafish model, however, did not protect cod, tilapia nor salmon.
Summary/Conclusion: The MVs from *P. salmonis* and *F. noatunensis* revealed a similar size distribution and that the content contains various bacterial virulence factors as well as DNA that can be transferred to the host. As for their immunogenic properties this seems to vary between the vaccine and challenge model compared to the natural hosts. The use of the MVs as vaccines in their natural hosts such as strain-specificity and cross-immunity need further investigation.
Funding: Research Council of Norway (RCN) and University of Oslo.

OF14.05

Bacterial membrane vesicles enter polarised epithelial cells and deliver their protein cargo to exosomes

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Introduction: Gram-negative bacteria use outer membrane vesicles (OMVs) to deliver a range of factors to host cells. Although OMVs are highly effective at entering simple non-polarised cell monolayers, it is not known whether these nano-sized vesicles can penetrate an intact epithelial barrier and, potentially, disseminate their protein cargo to tissues.
Methods: We have addressed this question using a cell culture model that reproduces the transepithelial resistance and apical-basolateral polarity of normal epithelium. For this, colonic epithelial cells of the T84 line were grown on Transwell filters to generate trans-epithelial electrical resistance (TEER), a measure of epithelial monolayer integrity. The cells were then co-cultured with Alexa Fluor-labelled OMVs from the gastric pathogen, Helicobacter pylori.
Results: We showed that H. pylori OMVs readily entered polarised epithelial cells, but had no effect on the TEER nor permeability of these monolayers. OMVs induced the basolateral secretion of the neutrophil chemoattractant, interleukin-8 (IL-8) and expression of human leukocyte antigen class I and II molecules. In exosomes isolated from the basolateral compartment of OMV-stimulated cells, we identified peptides derived from eight H. pylori proteins, of which seven are surface- or membrane-associated and are known to localise within OMVs.
Summary/Conclusion: Collectively, the data show that OMVs can enter polarised epithelial cells and deliver their protein cargo to exosomes. We propose that these exosomes may directly or indirectly present antigen to immune cells and even transport bacterial proteins to other tissue sites.
Funding: This project was supported by funding from the National Health and Medical Research Council (NHMRC), the Australian Research Council, The Juvenile Diabetes Research Foundation and the Victorian Government's Operational Infrastructure Support Program. R.L.F. is supported by an NHMRC Senior Research Fellowship. N.S. is funded through a Canadian MSFHR Research Trainee Fellowship and an NHMRC Early Career Fellowship. L.T. was funded by an Australian Postgraduate Award and an Excellence Award from Monash University FMNHS.

OF14.06

Bacterial Extracellular Vesicles: intercellular package or intracellular garbage? The example of RNAs associated to Salmonella enterica EVs

Antoine Malabirade[a], Janine Habier[a], Anna Heintz-Buschart[b], Patrick May[a], Julien Godet[c], Rashi Halder[a], Alton Etheridge[d], David Galas[d], Joëlle V. Fritz[a] and Paul Wilmes[a]

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Introduction: Bacteria have developed many ways of communicating with one another and with other prokaryotic or eukaryotic species. The secretion of Extracellular Vesicles (EVs) is one of them. Bacterial

EVs are small spherical containers filled with a wide range of biomolecules originating from the mother cell, including RNAs. The protection conferred by the physical envelope of EVs to these delicate components is of prime importance for message delivery to other cells. However, this idea of EVs being mail carriers competes with the concept of a simple trash bin used by bacteria to get rid of unnecessary components.

Methods: Taking Salmonella enterica as an example, we purified EVs and sequenced their RNA content. The strain was cultivated in different conditions mimicking separate stages of a gut infection. Growth until stationary phage in Lysogeny Broth (LB) medium induces Salmonella pathogenicity island 1 (SPI-1), which is required for virulence during the intestinal phase of infection. Growth in acidic and phosphate-depleted medium triggers the expression of Salmonella pathogenicity island 2 (SPI-2) and is comparable to the macrophage environment.

Results: Every type of RNA was exported, including ribosomal, messenger and non-coding RNAs. By comparison with the intracellular RNA composition, our data demonstrate that a proportion of RNAs exported through EV secretion were enriched. This export is depending on the environmental conditions and reflects the adaptation to each infection step. Some transcripts were confirmed to be in their native state and not degradation products, opening the possibility for a functional RNA delivery to surrounding cells. Finally, we show by a digestion protection assay that vesicles prevent enzymatic degradation of given full-length transcripts (SsrS, CsrC, 10Sa and rnpB).

Summary/conclusion: These results reinforce the idea of a complex interaction network existing in the gut microbiome and more generally in microbial ecosystems.

Funding: Luxembourg National Research Fund (FNR) (CORE Junior/14/BM/8066232, CORE/15/BM/10404093, CORE/16/BM/11276306), NIH Common Fund Extracellular RNA Communication Consortium (1U01HL126496), Baylor subaward (5U54DA036134).

Plenary Session 2: Therapeutics
Chairs: Edit Buzás; Uta Erdbrügger
Location: Level 3, Hall B

11:54–11:55

Self-assembled supramolecular nanosystems for Smart diagnosis and targeted therapy of intractable diseases

Kazunori Kataoka

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Nanotechnology-based medicine (Nanomedicine) has received progressive interest for the treatment of intractable diseases, such as cancer, as well as for the non-invasive diagnosis through various imaging modalities. Engineered polymeric nanosystems with smart functions play a key role in nanomedicine as drug carriers, gene vectors and imaging probes. This presentation focuses present status and future trends of supramolecular nanosystems self-assembled from designed block copolymers for therapy and non-invasive diagnosis of intractable diseases. Nanosystems with 10 to 100 nm in size can be prepared by programmed self-assembly of block copolymers in aqueous entity. Most typical example is polymeric micelle (PM) with distinctive core-shell architecture. PMs have several properties relevant for nanosystems, including controlled drug release, tissue penetrating ability, and reduced toxicity[1,2]. Furthermore, smart functionalities, such as pH- and/or redox potential responding properties, can be integrated into the PM structure[3]. These smart PMs loaded with various chemotherapy reagents were evidenced to have a significant utility in the treatment of intractable and metastatic cancers, including pancreatic cancer[4], glioblastoma[5] and tumours harbouring recalcitrant cancer stem cells (CSCs)[6]. Eventually, five different formulations of the PMs developed in our group have already been in clinical trials world-wide, including Japan, Asia, USA and European countries[7].

Versatility in drug incorporation is another relevant feature of supramolecular nanosystems for drug delivery. Nucleic acid-based medicine can be assembled into nanosytstems through the electrostatic interaction with oppositely-charged polycationic block copolymers[8]. In this way, siRNA- or antisense oligo (ASO)-loaded micellar or vesicular nanosystems were prepared, and their utility in molecular therapy of cancer has been revealed[9-11]. Recently, nanosystem-based imaging reagents were developed, opening a new avenue for the novel type of theranostic nanomedicines[12]. Furthermore, nanosysems hold promise for the treatment of intractable diseases other than cancer. Very recently, we developed nanosystems decorated with glucose to crossing blood-brain barrier by recognizing glucose-transporter overexpressing on brain endothelial cells, indicating a novel route to deliver versatile drugs into brain for the treatment of neurodegenerative diseases, including Alzheimer's disease[13].

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MSC-sEV translation: back to basics

Sai Kiang Lim

Institute of Medical Biology (IMB)

Mesenchymal stromal cell (MSC) are presently the most used cell type in clinical testing and are being tested against a wide spectrum of diseases. Their therapeutic efficacy is increasingly shown to be mediated by their secretion and in particular, the secreted extracellular vesicles (sEVs) of 50–200 nm. In this talk, I will elaborate on the development of clinical applications for MSC sEVs and the associated challenges. A major challenge is the complexity of a typical MSC-sEV preparation. As the size ranges of many EV types such as exosomes, microvesicles, and ectosomes overlap significantly and most EV types include EVs of 50–200 nm, the term "sEVs" essentially describes a complex population of similarly sized EVs consisting of many known

and possibly unknown EV types. This complexity is further compounded by the heterogeneity in the source and culture of MSCs, and in the downstream processing of MSC secretion. Together, this poses a challenge to data sharing by the research community and to the regulation of MSC sEVs as therapeutic products. Unfortunately, resolution of this conundrum through process standardization or purification of specific EV type is presently not practical and/or technically challenging. To circumvent this, about 20 members from the ISCT, ISEV, ISBT and SOCRATES have recently proposed several metrics to quantify distinctive features of a MSC-sEV preparation that will identify the cellular origin of the sEVs in a preparation, presence of lipid-membrane vesicles, and the degree of physical and biochemical integrity of the vesicles. Such metrics will facilitate comparison among different MSC-sEV preparation as differences could then be mapped to quantified differences in the features. The biological significance of such mapping would then be testable.

Featured Abstracts- Session 1
Chairs: Edit Buzás; Uta Erdbrügger
Location: Level 3, Hall B

11:55–12:30

FA1.01

Molecular basis for contradictive roles of melanoma-derived EVs in metastasis

Maximiliane Schuldner[a] and Elke Pogge von Strandmann[b]

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Introduction: Recent studies have highlighted the role of melanoma cell-derived EVs in the formation of pre-metastatic niches or, on the contrary, in tumour immune surveillance. The molecular machinery and mechanisms directing distinct cargo loading, regulatory release and function of stress-induced EVs remain unknown.
Methods: EV release was quantified by NTA. EVs were isolated by ultracentrifugation and analysed by proteomics and transcriptomics. EV function was investigated *in vivo* by intravenous injections followed by lung transcriptomics and by using an experimental metastasis transplantation model. The mechanistic release of EVs was analysed using diverse molecular, cell biological, spectroscopic and microscopic techniques.
Results: Our study reveals a crucial role of the chaperone and NK cell ligand BAG6 for the formation and reprogramming of pro- and anti-tumour EVs. Loss of BAG6 led to an increase in EV production and a decrease in EV size. In contrast to the melanosome-like protein signature observed for WT-EVs, BAG6KO-EVs showed an exosome-like profile and induced a neutrophil gene signature in the lungs of mice. Education with B-16V WT-EVs, but not BAG6KO-EVs, suppressed lung metastasis concomittant with the accumulation of anti-tumour Ly6Clow patrolling monocytes. Mechanistically, the formation of anti-tumour EVs was dependent on BAG6 mediating the nucleo-cytoplasmic shuttling of CBP/p300 acetyltransferases to acetylate p53. We have identified a late endosomal P(S/T)AP motif in BAG6 which mediated its direct recruitment to the ESCRT machinery, thereby providing a molecular link between the regulatory role of BAG6 to EV cargo loading.
Summary/Conclusion: Our findings provide a conceptual advance in the understanding of the biogenesis and function of EVs, identifying BAG6 as an ESCRT-associated protein and a molecular switch for the formation of anti- versus pro-tumourigenic EVs in tumour immune surveillance.

FA1.02

Development of a live-cell imaging technique for secretion activity of extracellular vesicles of individual cells

Yoshitaka Shirasaki[a], Keisuke Tsukada[b], Nobutake Suzuki[c], Tamiko Minamisawa[d], Mai Yamagishi[e], Nobuyoshi Kosaka[f], Takahiro Ochiya[f], Osamu Ohara[g], Kiyotaka Shiba[h] and Sotaro Uemura[i]

[a]JST PRESTO, Tokyo, Japan; [b]The University of Tokyo, bunkyo, Japan; [c]The university of Tokyo, Bunkyo-ku, Japan; [d]Japanese Foundation For Cancer Research, Koto-ku, Japan; [e]Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Japan; [f]Department of Molecular and Cellular Medicine, Institute of Medical Science, Tokyo Medical University, Shinjyuku-ku, Japan; [g]RIKEN Institute for Integrative Medical Sciences, Yokohama, Japan; [h]Japaese Foundation for Cancer research, Tokyo, Japan; [i]The University of Tokyo, Tokyo, Japan

Introduction: The cells in our body exchange their information using various methods to control the expression of functions, to form higher order systems and to maintain homeostasis. Particularly in the communication between spatially separated cells, mediation of humoral factors such as cytokines can be mentioned. Addition to this, extracellular vesicles (EVs) have been reported to participate in intercellular communication. The EVs classically include exosomes, microvesicles and apoptotic bodies. Also, vesicular autophagosome and transport vesicles in the intercellular space might be released during necrosis-like cell death (pyroptosis, necroptosis etc.). Although conventional biochemical methods can classify these EVs by size, density or antigens on their membrane, it is difficult to distinguish individual vesicles depending on their biogenesis.
Methods: We have developed LCI-S (Live Cell Imaging for Secretion activity), which is a time-resolved microscopic observation technology of secreted humoral factors from individual cells. LCI-S utilises sandwich fluoroimmunoassay and in situ detection of the immunocomplex by total internal reflection microscopy. LCI-S reveals intercellular variability of secretion activity and time-correlation with the cellular state such as intracellular enzymatic activity, cell shape or viability.

In this study, we focused on the release of EVs having the same topology as cell membranes such as exosomes, targeting membrane surface antigens such as CD63 and CD9.

Results: We have evaluated EVs secretion from kinds of cancer cell lines and succeeded in detecting vesicle-like adherent plaque under the cells and also free-diffusion type vesicles (free-EVs). We also found that the minor population showed higher secretion activity of free-EVs in our experimental condition. Furthermore, free-EVs secretion activity was not uniform nor constant. Some cells showed a burst secretion mode, and some showed an accelerated secretion mode. The cells bursting free-EVs tended to be associated with membrane blebbing, suggesting that they were in an excessive stress state.

Summary/Conclusion: In this study, we demonstrated that LCI-S has the potential to distinguish individual EVs depending on their biogenesis.

Funding: This research was supported by JST, PRESTO Grant Number JP17940748, Japan.

Symposium Session 15: EVs in Cancer
Chairs: Takahiro Ochiya; Carolina Soekmadji
Location: Level 3, Hall B

13:30–15:00

OF15.01

Transfer of functional cargo in exomeres

Qin Zhang[a], James Higginbotham[b], Dennis Jeppesen[a], Yu-Ping Yang[a], Wei Li[b], Ramona Graves-Deal[a], Jie Ping[a], Colleen Britain[c], Kaitlyn Dorsett[c], Celine Hartman[d], David Ford[d], Ryan Allen[a], Kasey Vickers[a], Qi Liu[a], Jeffrey Franklin[a], Susan Bellis[c] and Robert Coffey[a]

[a]Vanderbilt University Medical Center, Nashville, USA; [b]Vanderbilt University Medical Center, Nashville, USA; [c]University of Alabama at Birmingham, Birmingham, USA; [d]Saint Louis University School of Medicine, Saint Louis, USA

Introduction: There is an increasing appreciation that secreted nanoparticles are a heterogeneous mixture of distinct entities. One newly identified nanoparticle, exomeres (< 50 nm), were recently identified by the Lyden lab using asymmetric flow field-flow fractionation (AF4), a method that is not widely available. No known biological function has been assigned to these nanoparticles. In this study, we employed a simplified ultracentrifugation method to isolate and characterize subpopulations of exomeres and distinguish them from exosomes.
Methods: A two-step ultracentrifugation method was used to separate exomeres from exosomes. Purified exomeres were characterized by NTA, TEM, proteomics, lipidomics, DNA and RNA analysis Cell surface target sialylation by exomeres was measured by flow cytometry using fluorescence-labelled SNA lectin. Subpopulations of exosomes were purified by fluorescence-activated vesicle sorting (FAVS) and analysed for distinguishing cargos. Normal and neoplastic mouse colonic organoids were used for functional studies comparing exosome and exomere activities.
Results: Our analysis of the content of exomeres largely confirms what has been reported by Lyden and co-workers. We identify distinct functions of exomeres mediated by two of their cargos, the β-galactoside α2, 6-sialyltransferase 1 (ST6Gal-I) that α2,6- sialylates N-glycans, and the EGF Receptor (EGFR) ligand, amphiregulin (AREG). Functional ST6Gal-I in exomeres can be transferred to recipient cells resulting in hypersialylation of cell surface proteins, including β1-integrin. AREG-containing exomeres elicit prolonged EGFR and downstream signalling in recipient cells, modulate EGFR trafficking in mouse-derived colonic organoids, and dramatically enhance the growth of tumour colonoids.
Summary/Conclusion: This study describes a simplified method for exomere isolation and provides the first demonstration of transfer of functional cargo by exomeres and underscores the functional heterogeneity of secreted nanoparticles.

OF15.02

Exosomes secreted from senescent cells provoke chromosomal instability

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Introduction: Cellular senescence is the state of irreversible cell cycle arrest that can be induced by a variety of potentially oncogenic stimuli and is therefore considered to act as an important tumour suppression mechanism *in vivo*. However, cellular senescence is also associated with the increasing expression and secretion of inflammatory and pro-proliferative factors. This phenotype, termed the senescence-associated secretory phenotype (SASP), contributes to cancer development. In addition to inflammatory proteins, we reported that exosome secretion has dramatically increased in senescent cells, acting as harmful SASP factors. Recently, we found that senescence-associated non-coding RNAs (SA-ncRNA) are enriched in exosomes and these exosomes provoke chromosomal instability in normal cells.
Methods: Pre-senescent normal human diploid fibroblasts were rendered senescent by either serial passage, ectopic expression of oncogene or X-ray irradiation. Then we collected the exosomes secreted from young or senescent cells and checked the component of exosomes. To analyse the biological function of these exosomes, colony formation analysis and karyotype analysis were performed. Additionally, we manipulated SA-ncRNA to load into exosome using Exotic devise, then investigated the biological roles of them.

Results: We found that epigenetic de-regulation of genomic DNA induces the aberrant expression of non-coding RNA in senescent cells and SA-ncRNAs are enriched in exosomes secreted from senescent cells. Surprisingly, these exosomes cause anchorage-independent growth of normal cells and change the number of chromosomes. It is therefore possible that the overexpression of SA-ncRNA in old mice may eventually promotes tumorigenesis. These results indicate that senescence-associated epigenetic dysregulation is likely to contribute to tumour development not only through SASP but also exosomes during aging process.
Summary/Conclusion: Here we show a novel function of exosomes secreted from senescent cells on chromosomal instability. These data suggest that senescence-associated exosome secretion may contribute to age-related increase of cancer incidence.
Funding: PRESTO, JST.

OF15.03

Orthotopic neuroblastoma tumour model generating GFP-labelled extracellular vesicles (EV) reveals specific capture of GPF EV by monocytes/macrophages and mesenchymal cells in liver and bone marrow

Yves A. DeClerck[a], Laurence Blavier[b] and Rie Nakata[c]

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Introduction: EV released by tumours reaches target cells at distant sites. The study of their capture *in vivo* has been limited by methods relying on intravenous injection (i.v.) of EV isolated *in vitro*. Using human tumour cells producing GFP-labelled EV, we have examined the capture of tumour-derived EV in distant organs *in vivo*.
Methods: Luciferase expressing NB cell lines (SK-N-BE (2), CHLA-136, CHLA-255) were transduced with a lentivector targeting the GFP protein to the exosomal membrane (CMV-XP-GFP-EF1 aka XPack). The analysis of EV produced by XPack NB cells by differential ultracentrifugation followed by OPDG confirmed the presence of GFP in fractions containing exosomes. Mice orthotopically implanted with XPack NB cells were sacrificed at week 2, 4, 6 and 8, and the bone marrow (BM), liver, lung, kidney, and spleen were examined by FACS and immunofluorescence imaging (BM and liver) for the presence of GFP+ cells. The presence of the disialoganglioside 2 (GD2) was used to distinguish positive tumour cells from host cells having captured EV.
Results: Preliminary experiments with PKH67-stained NB-derived EV injected i.v. showed that after 24 h 0.9–1% of CD 45+cells in the BM, 6.7–20.3% of CD105 + cells in the bone, and 0.2–8.2% of CD45+ in the liver and lung contained green vesicles. In mice orthotopically implanted with NB cells producing GFP-labelled EV, we observed an increasing amount of GD2- /GFP+ cells in the BM (0.2%) between week 2 and 6. The expression of CD45, CD11b, and CD105 in these GD2- cells suggests their myeloid, monocytic, and mesenchymal origin. In the liver, a similar capture by CD45+ and CD11b+ was observed (up to 0.2%). We also observed an increasing amount of GD2- /GFP+ cells that were negative for CD45, CD11b, and CD105 at week 6–8. No GFP+ cells were detected in the lung, spleen and kidney.
Summary/Conclusion: Tumour-derived exosomes are specifically captured by a small percentage (within the limits of FACS detection) of myeloid and stromal cells in the BM and the liver in the early stages of tumour development before NB cells home to these organs. The data which used an orthotopic model rather i.v. injection, support the concept that exosomes contribute to the pre-metastatic niche.
Funding: RO1 CA 207983 from the National Institutes of Health, USA.

OF15.04

ExoBow – a transgenic strategy to study CD63+extracellular vesicles *in vivo*

Bárbara Adem[a], Nuno Bastos[a], Carolina Ruivo[a], Maxwell Goodrich[b], Zhang Xiaojing[c], Barbara Seidler[d], David W Goodrich[e], Jose L Costa[f], José Machado[f], Dieter Saur[g], Dawen Cai[h] and Sónia Melo[f]

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Introduction: The whereabouts of extracellular vesicles (EVs) inside a multicellular organism following their spontaneous natural flow and the identification of their recipient cells is still elusive. A comprehensive map of the network of communication established by EVs *in vivo* requires the development of new tools.

Methods: We have developed a CD63 multireporter transgenic mouse model to determine the spatiotemporal biodistribution of tissue/cell specific derived CD63-enriched EVs, exosomes, that we termed ExoBow. Using organ-specific promoters we have mapped the network of communication mediated by pancreas and intestine derived exosomes within the respective organ microenvironment, and also with neighbour and distant organs. The ExoBow transgene allows a stochastic Cre recombination that determines the expression of one of the fusion proteins CD63-mCherry, -phyYFP, -eGFP or -mTFP, and secrete colour-coded CD63+ EVs. We have used genetically engineered mouse models of pancreatic cancer crossed with our ExoBow to determine the flow of cancer exosomes during disease progression.
Results: We demonstrate that communication from the pancreas occurs more frequently upon cancer-associated transformation when compared to a healthy setting.
Summary/Conclusion: Our work is the first attempt to dissect the spontaneous flow of exosomes in a multicellular organism and to understand their involvement in several processes that occur in non-pathological and in pathological conditions. The ability of the ExoBow model to conditionally label any unique organ/tissue/cell within a mouse, opens an unprecedented opportunity to determine the connectome established by the flow of exosomes *in vivo*, unravelling their biological significance in health and disease.
Funding: NORTE-01-0145-FEDER-000029. Fundacao Ciencia Tecnologia: IF/00543/2013/CP1184/CT0004, PTDC/BIM-ONC/2754/201, POCI01-0145-FEDER-32189. FAZ Ciencia Astrazeneca

OF15.05

BMP2-dependent osteoblast differentiation is suppressed by multiple myeloma-derived extracellular vesicles

Mariko Ikuo[a,b], Kei Sugisaki[b], Jumpei Teramachi[b], Ryou-u Takahashi[a], Masahiro Abe[b], Kohji Itoh[b] and Hidetoshi Tahara[a]

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Introduction: Multiple myeloma (MM) suppresses osteoblast differentiation and destroys bones. Cancer-derived extracellular vesicles (EVs) such as exosomes control microenvironments, but little is known about EVs and exosomes secreted from MM cells (MM-EV). We examined whether and how MM-EV affects osteoblastic differentiation.
Methods: The mouse pre-osteoblast MC3T3-E1 cells and human osteosarcoma SaOS-2 cells was stimulated with bone morphogenic protein-2 (BMP2) to activate BMP/Smad pathway and induce osteoblastic differentiation. Alkaline phosphatase (ALP) induction and calcium deposition were used as indicators of differentiation. The promoter activities of Smad's target genes were quantified by luciferase reporter assays.
Results: In BMP2-treated MC3T3-E1, MM-EV repressed ALP induction and calcium deposition. MM-EV fractions were collected by Total Exosome Isolation Reagent (Invitrogen) or ultracentrifugation. The ALP suppression activity of the MM-EV collected by the kit and MM-EV collected by ultracentrifugation were correlated with the vesicle quantity and exosomal marker protein quantity. The suppression of ALP induction by MM-EV was inhibited by macropinocytosis inhibitor 5-(N-Ethyl-N-isopropyl) amiloride. In mouse cell MC3T3-E1 and human cell SaOS-2, MM-EV did not suppress Smad signal transduction. Contrary, these MM-EV inhibited promoter activation of genes targeted by Smad. This suppression activity required Smad binding elements (SBEs) of the promoter sequence. On Smad target promoters, a transcription factor X co-represses Smad's activity and inhibit osteoblast differentiation. The factor X was translocated in the nucleus and its target genes' expressions were changed in the cells treated with MM-EV.
Summary/Conclusion: MM-EV suppresses osteoblast differentiation by inhibiting promoter activation of Smad. This finding will lead a novel drug development strategy for the bone defects of MM.
Funding: Research Support Foundation of Tokushima University and TAIHO Pharmaceutical Co., LTD, JSPS Grant-in-Aid for Young Scientists (B) (ID 26860037), and JSPS Grant-in-Aid for Early-Career Scientists (ID 18K15213).

OF15.06

Tumour-derived extracellular vesicles require β1 integrins to promote anchorage-independent growth

Lucia R. Languino, Rachel DeRita, Aejaz Saeed, Vaughn Garcia, Shiv Ram Krishn, Christopher Shields, Andrea Friedman and Srawasti Sarker

Thomas Jefferson University, Philadelphia, PA, USA

Introduction: While the significance of extracellular vesicles (EVs) in disease progression is known, it is not clear whether "tumour-derived" EVs are detectable *in vivo* and are active. EVs contain different integrins; the β1 integrins, which are expressed in different cell types, contribute to cancer progression, and are known to signal through endosomes. In this study, we investigated whether prostate cancer (PrCa) EVs affect

anchorage-independent growth and whether β1 integrins in EVs are required for this effect.

Methods: We used EVs separated by ultracentrifugation and density–gradient from TRAMP mice, which develop PrCa (TRAMP, transgenic adenocarcinoma of the mouse prostate). We also used a cell line-based genetic rescue approach. For this study, we selected EVs with 1.14g/ml density and 100nm mean size.

Results: We show that EVs from either cancer cells *in vitro* or from blood of tumour-bearing TRAMP mice promote anchorage-independent growth of PrCa cells. In contrast, EVs from cultured cells harbouring a shRNA to β1, from wild-type mice or from β1pc-/-/TRAMP mice carrying a β1 conditional ablation in the prostatic epithelium, do not. Additionally, we show that genetic rescue of β1 restores the stimulatory function of secreted EVs on anchorage-independent growth. We demonstrate that EVs isolated through density–gradients from cancer cells or TRAMP blood, are functional and co-express β1, src, as well as CD9, CD63 and TSG101; in contrast, EVs from β1pc-/-/TRAMP or wild-type mice lack β1 as well as the other markers listed above.

Summary/Conclusion: In this study, we demonstrate that tumour-derived epithelial EVs require β1 integrins to stimulate anchorage-independent growth of recipient cells. Overall, this study opens new perspectives in cancer treatment based on inhibition of circulating β1 integrin- containing EVs shed by cancer cells.

Funding: This study was supported by NIH R01 CA-224769, P01 CA-140043; Thomas Jefferson University Dean's Transformational Science Award. This project is also funded, in part, under a Commonwealth University Research Enhancement Program grant with the Pennsylvania Department of Health (H.R.); the Department specifically disclaims responsibility for any analyses, interpretations or conclusions.

Symposium Session 16: Central Nervous System EVs
Chairs: Lesley Cheng; Dimitrios Kapogiannis
Location: Level B1, Hall A

13:30–15:00

OF16.01

Brain tissue-derived extracellular vesicles of Alzheimer's disease patients with different apolipoprotein E genotypes

Yiyao Huang[a], Vasiliki Machairaki[b], Lesley Cheng[c], Olga Pletniková[a], Juan Troncoso[a], Andrew Hill[d], Lei Zheng[e] and Kenneth W. Witwer[a]

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Introduction: Sporadic Alzheimer's disease (AD) associates with Apolipoprotein E (*APOE*) genotype. The ε4 allele is associated with increased risk vs. the more common ε3, while ε2 is protective. Recently, Vella, et al. (JEV, 2017) reported efficient enrichment of EVs from brain by differential and gradient density ultracentrifugation. Importantly, the method was carefully evaluated by levels of proteins presumed to be depleted in EVs vs. artefacts of tissue processing, per MISEV. Using a modification of this rigorous method, we extracted brain-derived EVs (bdEVs) of AD patients with different APOE alleles and non-AD brain tissues for quantitive and qualitative evaluation of EVs and their cargo.
Methods: Brain of AD patients with different APOE genotypes [ε2/ε3 (n = 5), ε3/ ε3 (5), ε3/ε4 (6), ε4/ε4 (6)] and non-AD controls (n = 7) was obtained from the Johns Hopkins Alzheimer's Disease Research Center. Tissue was processed per Vella et al. (JEV, 2017) through 10k x g centrifugation. Subsequently, SEC was followed by UC to concentrate bdEVs. Protein and particle concentration, morphology, and protein markers were examined by BCA, nano-flow cytometry (NanoFCM), TEM, and Western blotting. RNA and protein from brain homogenate (BH), 10k x g large EVs (lEVs) and small EVs (sEVs) were extracted for proteomics and small RNA QC (Fragment Analyser) and sequencing.
Results: bdEVs of acceptable purity were obtained using the modified method. No remarkable differences in bdEV morphology or size distribution were observed between AD and non-AD material. Similarly, no significant differences in particle counts separated AD from non-AD controls. Stratifying by APOE genotype several differences were observed. In contrast with a recent report on APOE4, counts of ε4-associated bdEVs were not lower than those of brains with other genotypes. Indeed, liberated particle counts were highest for ε4/ε4. Fragment Analyser revealed abundant sRNAs in sEVs. Total RNA and miRNA abundance from highest to lowest by source was: BH, lEVs, and sEVs.
Summary/Conclusion: Our results suggest ε4/ε4 genotype in AD associates with greater bdEV recovery than for other genotypes or non-AD brain. Ongoing evaluation of protein and RNA from these samples may reveal correlates or mechanisms of EV release.
Funding: US NIH: NIA (AG057430), NIMH (MH118164).

OF16.02

Murine CNS-Derived extracellular vesicles originate from astroctyes and neurons and carry misfolded proteins

Judith Maxwell. Silverman, Sarah Fernando, Catherine Cowan, Luke McAlary, Leonard Foster and Neil R. Cashman

University of British Columbia, Vancouver, 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 occurs at the organism level also. However, despite clear importance to the understanding of EVs in organismal biology, EVs in solid tissues have received little attention. Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease resulting in the progressive loss of motor neurons in the brain, brainstem and spinal cord. The disease is characterized by progressive propagation of pathology spreading from the CNS foci in which symptoms first appear.
Methods: To better understand to role of EVs in an ALS-affected central nervous system, we employed a method of whole tissue vesicle isolation. We applied a protocol for primary neural cell culture and modified it for the collection of EVs from frozen whole murine and human neural tissues by serial centrifugation and purification on a sucrose gradient.

Results: Quantitative proteomics found that brain-derived EVs contain canonical exosomal markers, with enrichment in synaptic and RNA binding proteins. The brain EVs contained numerous proteins implicated in ALS, and SOD1G93A transgenic EVs were significantly depleted in myelin-oligodendrocyte glycoprotein compared to non-transgenic animals. Brain and spinal cord EVs are positive for the astrocyte marker GLAST and the synaptic marker SNAP25, while CD11b, a microglial marker, was largely absent, suggesting that microglia do not contribute to the tissue EV population under these conditions. EVs from SOD1G93A transgenic ALS mouse model brains and spinal cords, as well as human SOD1 familial ALS patient spinal cord, possess abundant misfolded and non-native disulfide-crosslinked aggregated SOD1.
Summary/Conclusion: We established a phenotypic profile of vesicles from whole mouse brains and spinal cords, and investigated how model motor neuron disease modifies this phenotype. The data demonstrates that intra-organ CNS-EVs from disease affected animals and humans contain pathogenic disease-causing protein, and suggests that in the brain and spinal cord, astrocytes and neurons, as opposed to microglia, are the main source of EVs.
Funding: A Bernice Ramsay ALS Canada grant supported the work, along with funding from the Paul Heller Memorial Fund for JMS.

OF16.03

Investigating microvesicle motion on neuron surface through optical tweezers

Giulia D'Arrigo[a], Martina Gabrielli[b], Dan Cojoc[c], Giuseppe Legname[d] and Claudia Verderio[e]

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Introduction: Microvesicles (MVs) play an essential role in intercellular communication. Exposing adhesion receptors, they can interact with target cells and deliver complex signals. It has been shown that MVs also cover a crucial role in the spreading of pathogens in neurodegenerative disorders, but almost nothing is known about how MVs can transport messages moving in the extracellular microenvironment exploiting neuronal connections.
Methods: In order to investigate the interaction of MVs with the plasma membrane of neurons, MVs released from cultured astrocytes and isolated by differential centrifugation, were added to the medium of cultured hippocampal neurons. Using optical manipulation, single MVs in suspension were trapped by an infra-red laser collimated into the optical path of the microscope, and delivered to neuron surface. The MV-neuron dynamics were monitored by collecting bright-field images.
Results: Analysis of time-lapse recordings revealed that MVs efficiently adhered to neurons and about 70% showed a displacement along the surface of neurites. Interestingly, the MVs velocity (143 nm/sec) is in the same range of retrograde actin flow, which regulates membrane diffusion of receptors linked to actin. Accordingly, we found that MV movement is highly dependent on neuron energy metabolism. Indeed, only 33% of MVs were able to move on energy depleted neurons treated with rotenone. Moreover, inhibiting neuron actin cytoskeleton rearrangements (polymerization and depolymerization) with cytochalasin D, which binds fast growing end of actin, the percentage of EVs able to move on neuron surface was significantly reduced from 79% to 54%, revealing that neuronal actin cytoskeleton is involved in EV-neuron dynamics.

Unexpectedly, we found by cryo-electron microscopy that a subpopulation of MVs contains actin filaments, suggesting an intrinsic capacity of MVs to move. To address this hypothesis, we inhibited actin rearrangements in EVs with Cytochalasin D and observed a significant decrease, from 71% to 45%, of MVs able to drift on neuron surface.
Summary/Conclusion: Our data support two different way of MV motion. In the first case, MV displacement could be driven by the binding with neuronal receptors linked to the actin cytoskeleton. In the second, actin rearrangements inside MVs could drive the motion along a gradient of molecules on neuron surface.

OF16.04

P2RX7 Inhibitor suppresses tau pathology and improves hippocampal memory function in tauopathy mouse model

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Introduction: Microglia, the innate immune cells in the central nervous system, could spread pathogenic tau protein via secretion of extracellular vesicles, such as exosome. P2X7 receptor (P2RX7) is an ATP-gated cation channel and highly expressed in microglia and triggers exosome secretion. We hypothesize that P2RX7 inhibitor could alleviate tauopathy in PS19 tau transgenic mice by inhibiting the exosome secretion by microglia.

Methods: BV-2 murine microglial cell lines were treated with GSK1482160, a specific inhibitor of P2RX7, prior to ATP stimulation. Exosomes were enriched from the conditioned media and quantified using Nanoparticle Tracking Analysis and CD9 ELISA. Three-months old P301S Tau (PS19) and control wild-type mice were treated with GSK1482160 (20 mg/kg) or vehicle by oral gavage for 30 days. The animals were tested for hippocampal memory function. The accumulation of pathogenic Tau was determined by immunohistochemistry and ELISA.

Results: ATP stimulation of BV-2 cells significantly increased secretion of exosomes (30–150 nm), which was significantly inhibited by GSK1482160 treatment in a dose-dependent manner. Daily administration of GSK1482160 over 30 days had no effect on body weight of PS19 mice. Interestingly, GSK1482160 treatment enhanced spontaneous alteration in Y-maze and improved prepulse inhibition as compared to vehicle-treated group. In addition, pTau level in the hippocampal tissue was significantly reduced in GSK1482160-treated PS19 mice as compared to vehicle-treated group as determined by neuropathology and ELISA.

Summary/Conclusion: GSK1482160 treatment suppresses ATP-induced exosome secretion from BV-2 cells. Its oral administration to PS19 mice improved hippocampal memory function and reduced the accumulation of pathogenic tau. These data demonstrate that targeting P2RX7 is a novel target for suppressing the exosomal spread of pathogenic tau protein by microglia, which may be applicable to Alzheimer's disease.

Funding: Grant/Other Support: NIH 1RF1AG054199-01

Grant/Other Support: 1R01AG054672-01
Grant/Other Support: 1R56AG057469-01
Grant/Other Support: DVT-14–320835
Grant/Other Support: Cure Alzheimer's Fund.

OF16.05

Astrocyte-derived extracellular vesicles shed in response to IL-1beta up-regulate amyloidogenic processing in neurons

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Introduction: Chronic inflammation is thought to contribute to the pathogenesis of Alzheimer's disease by upregulating amyloidogenic processing of APP. Based on previous findings that inflammatory stimuli modify the cargo of astrocyte derived extracellular vesicles (ADEV), we sought to determine if ADEVs released in response to IL-1β (ADEV-IL-1β) contain cargo that regulate APP processing in neurons.

Methods: Neurons were stimulated with constitutively released ADEV or ADEV-IL-1β. APP, and BACE1 co-localization in membrane microdomains was measured by immunofluorescence staining. APP and BACE1 protein and mRNA levels measured by western blot and RT-QPCR. The binding of APP mRNA and heterogeneous nuclear ribonuclear protein C (hnRNP C) was measured by immunoprecipitation. Casein kinase 1 (CK1) efficiency was blocked using pharmacological inhibition and genetic knockdown. Isolation of human plasma ADEV was achieved by GLAST-1 pull-down.

Results: Neurons exposed to ADEV-IL-1β promoted the co-localization of APP and BACE1 into membrane microdomains ($p < 0.001$), and increased production of Aβ1-42. Protein expression of APP ($p = 0.0051$) but not BACE1 was also increased. APP mRNA was not increased following exposure of neurons to ADEV-IL-1β, suggesting a post-translational event. APP translation is regulated by a competitive interaction of the fragile X mental retardation protein (FMRP), and hnRNP C with the APP coding region. hnRNP C can displace FMRP and increase APP translation. The association of hnRNP C with APP mRNA increased following exposure to ADEV-IL-1β. Compared with ADEV-CR, ADEV-IL-1β selectively carried CK1. CK1 is known to activate hnRPC. Knockdown of CK1 in astrocytes reduced CK1 in ADEV-IL-1β ($p = 0.0061$), prevented the upregulation of APP protein expression ($p = 0.007$), and co-localization of APP with BACE1 ($p < 0.001$). Overexpression of CK1 in astrocytes increased CK1 in ADEV-IL-1β, increased APP protein expression ($p = 0.0026$), and co-localization of APP with BACE1 ($p = 0.0015$). We confirmed in ADEVs isolated from human plasma that AD patients, but not healthy age-matched controls contain CK1 ($p = 0.0467$).

Summary/Conclusion: These data suggest that neuroinflammatory stimuli modify ADEV cargo to enhance amyloidogenic processing of APP by delivering CK1 to regulate the association of hnRNPC with APP mRNA.

OF16.06

The role of human choroid plexus-derived extracellular vesicles in viral neuroinvasion

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Introduction: The human polyomavirus JCPyV causes the fatal disease progressive multifocal leukoencephalopathy

(PML) in immunocompromised individuals and patients undergoing immunomodulatory therapy. A critical question in JCPyV pathogenesis is understanding how the virus is transported from the periphery to the CNS to infect glial cells and cause demyelination. An additional paradox is that the target glial cells do not express known virus receptors. Previously, we analysed JCPyV infection of the choroid plexus (CPE), a functional barrier to the CSF and showed CPE cells are permissive to JCPyV infection and express viral attachment and entry receptors. Here, we investigate the role of CPE-derived extracellular vesicles in receptor-independent infection of glial cells by JCPyV.

Methods: In addition to analysing primary human CPE cells, we also developed an immortalized human CPE line. CPE were transformed using hTERT lentiviral transduction and verified by STR profiling. EV from both cell types were concentrated by differential centrifugation and evaluated by transmission electron microscopy, Western blot, nanoparticle tracking analysis, infection, and qPCR for protected viral genomes. Infection was evaluated by immunofluorescence analysis with antibodies against the major viral capsid protein VP1. Uptake was evaluated by flow cytometric analysis of PKH67 fluorescently labelled particles and confocal imaging.

Results: EV from CPE cells display characteristic markers and morphology, contain intact JCPyV virions, and are infectious to both CPE cells and human glial cells. EV-mediated infection is receptor independent. Infection and uptake of EV cannot be inhibited by neutralizing antisera and internalization is via endocytosis. EV from CPE contains significantly more VP1 than other glial cell lines.

Summary/Conclusion: Primary and transformed CPE produce similar EVs that are able to deliver significant amounts of JCPyV in a receptor independent manner to target cells in the CNS. The choroid plexus may be an entry point by which JCPyV accesses the brain leading to the development of PML.

Funding: NIH RO1NS043097.

Symposium Session 17: EVs in Tissue Injury and Repair
Chairs: Benedetta Bussolati; Dominique de Kleijn
Location: Level B1, Hall B

13:30–15:00

OF17.01

Mesenchymal stem cell derived extracellular vesicles restore the engraftment capacity of stem cells in radiation exposed mice

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Introduction: We have shown that pretreated irradiated murine bone marrow stem cells (BMSCs) with mesenchymal stem cells-extracellular vesicles (MSC-EVs) *in vitro*, could significantly improve the engraftment capacity of radiation damaged BMSCs with a predominant reversal effect in later periods of post-transplant from 12 weeks up to 36 weeks. This indicates a long-term effect of MSC-EVs on reversal of radiation damage of BMSCs.
Methods: In this study, we investigated the long-term effect of MSC-EVs on the restoration of engraftment of BMSCs in radiation-exposed mice *in vivo* up to 53 weeks. Moreover, the safety and toxicity of MSC-EVs treatment were also evaluated.
Results: 500cGy radiated mice were injected with human MSC-EVs by tail vein injection at 24, 48 and 72h post-radiation. We followed the peripheral blood cell counts up to 53 weeks post-EV injection. There was a significant RBC, HGB and platelet restoration in EV treated radiated mice compared to untreated mice in the early period (before day 35). For the evaluation of reversal effect on BMSCs, bone marrow, harvested at 6, 12, 26 and 53 weeks. post-EV injection, were transplanted into 950 cGy exposed B6.SJL mice. The engraftment was evaluated at 4 and 12 weeks post-transplantation. In those transplanted mice at 6 weeks post-EV injection, there was a slight increase in the restoration of engraftment rate in EV treated mice (17.58 ± 2.32%) compared to untreated mice (13.80 ± 1.41%) after 1 month post-transplantation. However, for those mice transplanted at 12, 26, and 53 weeks post-EV injection, there were significantly higher restorations of engraftment in EV treated mice (40.48 ± 6.03%, 33.93 ± 3.76%, and 56.62 ± 3.63) compared to untreated mice (12.39 ± 1.30%, 15.14 ± 2.21%, 36.21 ± 3.63%) after 4 weeks transplantation, respectively. The similar restorations of engraftment were also seen in 12 weeks post-transplantation. These data suggested that EVs have early and late mitigating effects on peripheral blood cytopenias and BMSCs. No toxic effect was observed in bone marrow, kidney, liver, spleen, lung and heart up to 53 weeks post-EV injection.
Summary/Conclusion: Our data suggest that there is a long-term effect of MSC-EVs on the restoration of engraftment of BMSCs in radiation-exposed mice, and MSC-EV treatment is a safe therapeutic strategy.
Funding: NIH grants 5UH2TR000880 and 5T32HL116249.

OF17.02

Connexin43-positive exosomes released by osteoarthritic chondrocytes favours osteoarthritis progression by spreading senescence and inflammatory mediators to nearby tissues

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Introduction: Chondrocytes in articular cartilage undergo phenotypic changes and senescence, restricting cartilage regeneration and favouring osteoarthritis (OA) progression. Like other wound healing disorders, chondrocytes from OA patients show a chronic increase in the transmembrane channel protein connexin43 (Cx43). Extracellular vesicles (EVs), including exosomes, have been show to harbour connexin

channels that allow the formation of gap junctions between the exosome and the target cell. However, the role of these vesicles and exosomal-Cx43 in OA progression has not been studied yet. The objective of this study was to investigate the role of EVs released by osteoarthritic chondrocytes (OACs) in cellular plasticity and senescence of surrounding tissues.

Methods: EVs were isolated from OA/healthy chondrocytes by ultracentrifugation and their protein content was analysed by LC-MS/MS using 6600 triple TOF. RNA levels, protein activity and cellular senescence were analysed by RT-qPCR, western blot, immunofluorescence and flow cytometry.

Results: Our results indicate that OACs contain increased levels of Cx43 within their EVs in comparison to the EVs isolated from healthy donors. Overexpression of Cx43 in chondrocytes increased senescence and the total content of Cx43 in the EVs. The treatment of target cells with EVs containing Cx43 led to a significant increase in Cx43 mRNA and protein levels. The increase of Cx43 lead to dedifferentiation in the recipient cells via EMT by activation of Twist-1, with increased levels of the mesenchymal markers CD105 and CD166. The phenotypic changes detected in OACs lead to a decrease in the main cartilage markers Col2A1 and ACAN expression, and increased the levels of cellular senescence and SASP in target cells via p53/p16 and NF-kß. These results were corroborated by analysing the protein cargo of these Cx43 positive EVs, where we found enrichment in proteins related with the catabolic, senescence and wound-healing pathways

Summary/Conclusion: Together, these results suggest that Cx43-positive EVs released by OACs may be involved in the spread of cellular senescence, inflammation and reprogramming factors involved in wound healing failure to neighbouring tissues in the joint. Further understanding of the role of exosomal Cx43 in OA will help to halt the disease spread and progression.

OF17.03

Extracellular vesicles in ageing: from skin to bone

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Introduction: Cellular senescence has evolved from an *in vitro* model system to study aging to a multifaceted phenomenon of *in vivo* importance since senescent cells *in vivo* have been identified and their removal delays the onset of age-associated diseases in a mouse model system. In order to understand how senescent cells that accumulate within organisms with age negatively impact on organ and tissue function, we have started to characterize secreted miRNAs within extracellular vesicles that are differentially expressed in early passage versus senescent cells and their functional role in the context of cellular and organismal aging.

Methods: We performed next generation sequencing as well as qPCR on extracellular vesicles from senescent versus control cells, after characterizing the EVs in detail. In addition, open flow microperfusion experiments were used to proof the presence of EVs in the interstitial fluid of the skin.

Results: We identified extracellular vesicle contained miRNAs as bona fide members of the senescence associated secretory phenotype (SASP) that are transferred from senescent cells to their microenvironment or even the systemic environment. These miRNAs, among them miR-23a-5p, are transported via extracellular vesicles also in organotypic human skin equivalents and recipient cells taking them up are altered in their cell fate, including altered wound healing and apoptotic behaviour. In addition, miR-31 is transferred to mesenchymal stem cells, inhibiting osteogenic differentiation.

Summary/Conclusion: In summary, we present evidence of the importance of specific miRNAs and highlight their potential use as biomarkers of aging and age-associated diseases, or even as therapeutic tools and targets to prevent age-associated diseases.

Funding: This work was funded by the Christian Doppler Society. The financial support by the Austrian Federal Ministry of Economy, Family and Youth; the National Foundation for Research, Technology and Development is also gratefully acknowledged, as is funding by the Austrian Science Fund (FWF: I2514 to JG) and the PhD Programme BioToP – “Biomolecular technolgy of proteins”.

OF17.04

Human embryonic stem cells derived exosomes promote tissue regeneration in aged mice by rejuvenating senescent endothelial cells

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Introduction: Angiogenesis plays a crucial role in tissue repair. This process is significantly impaired by age-related dysfunction of vascular endothelial cells in aged bodies. Exosomes from embryonic stem cells (ESCs) contain primitive molecules (proteins, miRNA, etc.) from their parent cells. Therefore, our hypothesis is that ESCs derived exosomes (ES-Exos) would influence and rejuvenate aging endothelial cells and lead to enhanced tissue repair in aged bodies.
Methods: Six- to eight-week-old C57BL/6 mice were daily subcutaneous injection of D-gal (1000 mg/kg) to establish aged mice model. Pressure ulcers were created on the back of each mouse, followed by pipetting ES-Exos (1*1011/mL) suspension or PBS one time per day. Mice were sacrificed at 3, 7, 14, and 21 days after intervention. In addition, a group of young mice with pressure ulcer was also set. Samples from each mouse were evaluated in the aspect of vascular formation and aging condition. Furthermore, we induced HUVEC senescence *in vitro* by D-gal treatment and investigate the function and mechanism of ES-Exos in restoring function and rejuvenation of senescent endothelial cells by qRT-PCR, WB, and immunofluorescent staining.
Results: Our results showed that ES-Exos treated aged mice exhibit faster repairing than PBS treated group. The angiogenesis condition of ES-Exos treated group was similar as that of young mice and was better than PBS treated senescent mice. The number of SA-β-gal-positive cells and the expression level of P16 and P21 in ES-Exos treated group were significantly lower than that in PBS treated group. *In vtiro* experiments showed that ES-Exos could also downregulate senescent related protein expressions and enhance tube formation of senescent endothelial cells. In addition, our results also showed that ES-Exos could greatly decreased the expression level of MDA and increase the activity of SAD, CAD, and GSH, molecules tightly related with endogenous anti-oxidative condition. Further investigation demonstrated that ES-Exos could activate NRf2 pathway by inhibiting Keap1, leading to rejuvenative function on senescent endothelial cells.
Summary/Conclusion: We demonstrate that ES-Exos can accelerate wound healing and promote angiogenesis in aged mice by rejuvenating endothelial senescence.
Funding: NSFC Project No. 81871833 and 81672254.

OF17.05

Schwann cell derived exosomes regulate Schwann cell activation and neuropathic pain related behaviours

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Introduction: Exosomes (Exs) are small extracellular vesicles originally known to be secreted from multivesicular endosomes in dendritic cells. We now know that Exs are secreted from many cell types and are essential for autocrine/paracrine communication. In the peripheral nervous system (PNS), Exs derived from primary Schwann cells (SC) appear to facilitate axon growth after injury, however their effects on SC physiology and pain outcomes are unknown.
Methods: Exs were purified from primary SC conditioned media by ultracentrifugation (SC-Ex) and characterized by immunoblotting and NanoSight In cultures of SC, TNFa robustly activated proinflammatory cell signalling and migration. SC-Ex (50–500 ng/mL) were added to TNFa treated SC, and phosphorylation of p38MAPK and JNK1/2 were measured. Transwells were used to evaluate SC migration. To determine if SC-Ex regulate neuropathic pain, we performed intraneural injections of SC-Ex (500–1500 ng) or vehicle into sciatic nerves during partial nerve ligation (PNL) surgeries in adult male rats ($n = 12$). Tactile allodynia was assessed using von Frey filaments.
Results: Nanoparticle tracking of SC-Ex showed the expected size distribution with a mean peak diameter of 121 nm. Immunoblotting of SC-Ex revealed that exosome markers, TSG101 and flotillin-1, and SC marker, P0 protein, were expressed. The golgi marker, GM130, and GFAP were not. In cultured SC, the SC-Ex signalling response was distinguished from the cell signalling signature elicited by TNF alone, which robustly activated p38MAPK and JNK1/2 by > 6 and 4-fold ($p < 0.01$), respectively. When SC-Ex were added, p38MAPK and JNK1/2 activation were dose dependently and significantly inhibited ($p < 0.05$). TNF increased SC migration 3-fold after 4 h that was blocked by SC-Ex at low doses. Local injections of SC-Ex modified tactile allodynia associated with PNL compared to saline injected controls.
Summary/Conclusion: We demonstrated that SC utilizes autocrine secretion of Exs for regulating SC signalling and migration. SC-Ex act as cell independent entities, carrying bioactive substances capable of inhibiting pro-inflammatory signalling in SCs that may contribute to the extent and magnitude of chronic pain. Future studies will elucidate SC-Ex cargo driving autocrine/paracrine activities after PNS injury.
Funding: VA.

OF17.06

Urinary extracellular vesicles improve the recovery of renal function in an Acute Tubular Injury model restoring Klotho levels

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Introduction: Extracellular vesicles present in urine (uEVs), are considered a non-invasive source of information regarding the pathophysiology of the whole kidney. Mainly secreted by renal cells lining the nephron, uEVs have been studied as biomarkers for diagnosis of renal diseases. However, their possible therapeutic use has not been addressed yet. In the current study, we investigated the potential therapeutic effect of uEVs, in a murine model of acute kidney injury (AKI). While the beneficial effect of mesenchymal stromal cell-derived EVs (MSC EVs) for AKI treatment has been extensively described, we here tested the possible therapeutic use of uEVs as more "renal committed" source.

Methods: uEVs were isolated by ultracentrifugation of human urine provided by healthy subjects. AKI was performed by intramuscular injection of 8 ml/kg hypertonic glycerol. Next day, 2 × 108 uEVs /mouse were intravenously injected and 48 h later mice were sacrificed.

Results: Our data showed that administration of uEVs in AKI mice resulted in the acceleration of renal recovery in a MSC EV-treatment comparable manner. Functional and histological abnormalities, observed upon AKI, were alleviated, cell proliferation was stimulated, while the expression of renal tissue injury and inflammation markers was reduced. The analysis of uEV miRNA cargo showed the presence of several miRNAs possibly involved in tissue repair. miR-30 and miR-151, previously described present in MSC EVs, were further found transferred in renal tissue of uEV-injected mice. In addition, the reno-protective factor Klotho, was found present in uEVs at both protein and mRNA level. The administration of uEVs in AKI mice resulted in the restoration of Klotho protein levels in renal tissue, significantly lowered upon damage. Of interest, ineffective fibroblast-derived EVs loaded with recombinant Klotho exhibited a reno-protective effect, suggesting a possible Klotho-mediated mechanism in the amelioration of AKI.

Summary/Conclusion: Overall, our results reveal a novel potential therapeutic approach for AKI treatment, using renal cell-derived EVs present in urine and indicate common, as well as unique, uEV and MSC EV mechanisms of action.

Funding: FP7 NephroToolsproject and by Miur ex60%

Symposium Session 18: EV Function in Health and Disease
Chairs: David Carter; Jacky Goetz
Location: Level B1, Lecture Room

13:30–15:00

OF18.01

Increased levels of systemic LPS-positive bacterial extracellular vesicles in patients with intestinal barrier dysfunction

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Introduction: Bacterial extracellular vesicles (EV) are secreted by gut bacteria and contain nucleic acids, proteins, metabolites and endotoxins. Consequently, bacterial EV that enters the systemic circulation may deliver and elicit a variety of immunological and metabolic responses in different organs. However, the systemic presence and activity of bacterial EV in patients with intestinal barrier dysfunction have not been investigated.

Methods: Size exclusion chromatography and density gradient centrifugation were combined to fractionate faeces and plasma in two dimensions separating bacterial EV-associated LPS from other LPS products and eukaryotic EV. Bacterial EV-associated LPS levels of 49 subjects with a compromised or intact intestinal barrier were measured by Limulus Amebocyte Lysate and Toll-like receptor four reporter assays and confirmed by immunoelectron microscopy. Plasma zonulin was measured to assess intestinal barrier integrity and Caco-2 transwell systems were used for *in vitro* validation experiments.

Results: We calculate that the human gut harbours approximately 100 trillion bacterial EV which may serve as a substantial source of systemic pathogen-associated molecular patterns (PAMP), evidenced by proteomic analysis of faeces-derived bacterial EV. We demonstrate that bacterial EV can translocate the intestinal epithelial layer in a paracellular way. LPS-positive bacterial EV levels are significantly increased in plasma of patients diagnosed with HIV, inflammatory bowel disease and cancer therapy-induced intestinal mucositis compared to respective controls (Mann-Whitney U test, $p < 0.01$). These bacterial EV are able to induce immune activation and significantly correlate with impaired barrier integrity of the patient (Spearman's $\rho = 0.4241$, $p = 0.0245$).

Summary/Conclusion: Pathologies with an intestinal barrier dysfunction open the door for bacterial EV to enter the circulation and to induce immune activation. Their systemic presence correlates with impaired gut barrier integrity. These data deliver novel opportunities to advance our knowledge of PAMP-induced systemic reactions and biomarker development.

Funding: This work was supported by concerted research action from Ghent University and Krediet aan Navorsers from the Research Foundation Flanders (FWO). JVD, EG, LV and AH are supported by fellowships from FWO.

OF18.02

Milk exosomes accumulate in the intestinal mucosa and peripheral tissues in wild-type pups nursed by exosome and cargo tracking dams

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Introduction: Exosomes and their cargos may be obtained from dietary sources such as milk. Hypothesis: Milk exosomes accumulate in the intestinal mucosa and peripheral tissues in suckling mice. Aims: 1) Develop an Exosome and Cargo Tracking (ECT) mouse to assess the origin, destination and cargo of exosomes. 2) Assess the bioavailability and distribution of milk CD63-positive EVs ("exosomes") labelled with fluorescent proteins in suckling pups.

Methods: ECT mice were developed by random integration of an ECT plasmid. The plasmid encodes a fusion protein of CD63 and green fluorescent protein (eGFP) plus stop codon, flanked by two loxP sites (ORF-1). A second ORF, coding for a CD63/near-infrared protein (iRFP) fusion protein, follows downstream of ORF-1. A transmembrane domain is fused to the C-terminus of the iRFP

to create an extra-exosomal C-terminus, followed by a second iRFP and a stop codon. In the presence of Cre, the CD63/eGFP/Stop insert is removed and the mice switch from expressing eGFP-labeled exosomes to iRFP-labeled exosomes, including an extra-exosomal iRFP for collection with anti-iRFP and magnetic beads. Wild-type (WT) pups were fostered to ECT dams to assess the bioavailability of milk exosomes.

Results: ECT mice showed no disease phenotypes. In the absence of Cre, the mice expressed eGFP-labeled exosomes, whereas the offspring of mice mated with Cre mice expressed iRFP-labelled exosomes. The same patterns were obtained when HEK-293 cells were transfected with the ECT plasmid in the absence of presence of Cre plasmid. Fluorescent proteins localized to exosomes but not to other complexes secreted by HEK-293 cells. The particles had the size expected for exosomes (131 ± 49 nm), stained positive for CD9, Alix and TSG101, stained negative for histone H3, and were captured by anti-CD63. When WT pups were nursed by ECT dams for three weeks, exosomes accumulated primarily in the intestinal mucosa, brain and kidneys.

Summary/Conclusion: We have developed a mouse that permits tracking CD63-positive exosomes and their cargos. CD63-positive exosomes are bioavailable in suckling mice.

Funding: NIFA, NIH, Gates Foundation, Gerber Foundation, USDA. JZ serves as consultant for PureTech Health, Inc.

OF18.03

Deciphering extracellular vesicle mediated host-pathogen interaction in streptococcus pneumoniae

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Introduction: Extracellular vesicles (EVs) represent a highly sophisticated cell-to-cell mailing system. Both eukaryotic and prokaryotic cells selectively pack signalling cargo into and onto EVs and deliver them to the extracellular milieu. However, it was not until recently, that Gram-positive bacteria including the major respiratory pathogen *Streptococcus pneumoniae* (pneumococcus), were shown to produce EVs, likely originating from the plasma membrane to be released into the extracellular milieu. The role of Gram-positive EVs in cell-to-cell communication, and their interactions with the host immune system remain poorly understood. Similarly, the interaction of eukaryotic immune EVs with pneumococcus remains unexplored. In this study, EVs from pneumococci and pneumococci-conditioned macrophages were isolated and reciprocal interaction between the host and the microbe was explored.

Methods: EVs from pneumococci and pneumococci-conditioned macrophages were isolated using size exclusion chromatography and characterized by transmission electron microscopy, dynamic light scattering, tunable resistive pulse sensing and western blotting. Effects of pneumococcal EVs on different immune cell subsets were assessed using flow cytometry, western blotting and immunostaining. Effects of EVs on S. pneumoniae were assessed by evaluating their growth kinetics and biofilm development.

Results: Pneumococcal EVs were internalized by various immune cells and conversely, eukaryotic EVs from J774A.1 cells were taken up by both Gram-positive and Gram-negative bacteria. In a reporter murine macrophage cell line (RAW 264.7), pneumococcal EVs induced NF-kB expression in a dosage-dependent manner and induced p65 nuclear translocation in human primary macrophages. Pneumococcal EVs also activated primary human CD4+ and CD8 + T cells as evidenced by their CD69 upregulation in a dose and time-dependent manner. Moreover, EVs secreted by S. pneumoniae promoted their own planktonic growth and biofilm development in a dose-dependent manner.

Summary/Conclusion: For the first time, we show that eukaryotic EVs are taken up by Gram-positive bacteria. Our data also indicate that pneumococcal EVs have immunomodulatory effects on the host immune cells and represents a sophisticated communication system that needs further investigation.

OF18.04

Calpain carried by platelet-derived microparticles mediates protease-activated receptor 1-dependent vascular inflammation in diabetes.

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Introduction: Diabetes mellitus is a major risk factor for cardiovascular diseases and platelet hyperactivation in diabetes is linked to the release of platelet-derived microparticles (PMPs) that carry the Ca^{b+}-activated protease calpain 1 (CAPN1). Here we determined whether CAPN1 could target proteins on the vascular wall that could precipitate the development of vascular disease.

Methods: Mass spectrometry and ELISA were used to analyse proteins in the culture medium. Protein levels on the surface of endothelial cells were measured by FACs and en-face immunostaining was used to assess protein levels on intact aorta. Diabetes was induced with streptozotocin.
Results: *In vitro* treatment of human 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. In agreement, EPCR levels were increased in plasma from diabetic patients. Also, diabetes induction in mice led to a similar increase in plasma EPCR levels, an effect prevented by treatment with the calpain inhibitor. At the molecular level, CAPN1 did not directly target the EPCR but rather cleaved the protease-activated receptor 1 (PAR-1), inducing an intracellular signalling cascade i.e the phosphorylation of protein kinase C (PKC) and the extracellular regulated kinase (ERK) as well as the activation of the tumour necrosis factor (TNF)- converting enzyme (TACE). The latter was responsible of EPCR shedding as well as of the increased local TNF- levels. TNF-triggered the phosphorylation of the p65 subunit of NFκB, increased ICAM-1 expression and enhanced monocyte adhesion. The same phenomenon was evident *in vivo* as en-face preparations of aortae from diabetic mice revealed a loss of PAR-1 but induction of ICAM-1 which could be prevented by CAPN inhibitor or by specific knocking out of CAPN1 in platelets (PF4-Capn1-/-). All of the effects of PMPs or CAPN1 were abolished in PAR-1-deficient endothelial cells.
Summary/Conclusion: These data demonstrate that platelet-derived calpains contribute to diabetes-associated vascular inflammation by targeting the PAR-1 receptor.
Funding: Deutsche Forschungsgemeinschaft RA 2435/3-1.

OF18.05

Plasma-derived extracellular vesicles from P. vivax patients increase ICAM-1 expression of human spleen fibroblasts facilitating adherence of infected reticulocytes

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Introduction: Using a reticulocyte-prone rodent malaria model, resembling P. vivax, our group demonstrated cytoadherence of infected reticulocytes to spleen blood barrier cells of fibroblastic origin (Martin-Jaular et al., 2011). Here, as extracellular vesicles (EVs) play a role in intercellular communication, we hypothesized that plasma-derived EVs from natural vivax infections (PvEVs) signal human spleen fibroblasts facilitating adherence of P. vivax, a reticulocyte-prone human malaria parasite.
Methods: Upregulation of ICAM1 and other targeted genes upon uptake of PvEVs in human spleen fibroblasts (hSF) was determined by qRT-PCR. Expression of ICAM1 was validated by FACS. NF-kB nuclear translocation analysis was determined by confocal microscopy. The binding capacity of P. vivax-infected reticulocytes from infections upon uptake of PvEVs was tested after maturation and purification of frozen estabilates of isolates from Mae Sot (Thailand). P. vivax-infected reticulocytes were incubated with hSF previously stimulated with PvEVs, hEVs or PBS, and the number of binding parasites determined by microscopy.
Results: ICAM-1, a known receptor for binding of malaria, was specifically upregulated by EVs from infections in a dose-dependent manner at mRNA and protein levels. NF-κ B was observed both in the cytoplasm and the nucleus on non-stimulated and hEVs-stimulated hSF, whereas PvEVs stimulation induced nuclear translocation of NF-κ B on hSF. By comparing the binding of iRBCs to hSF, we last demonstrated significant higher binding to the cells after uptaken of exosomes from infections.
Summary/Conclusion: These results suggest that circulating exosomes from vivax malaria infections have spleen-tropism signalling spleen fibroblasts to induce ICAM-1 through NF-kB and facilitate adherence of infected reticulocytes. Thus, unveiling molecular insights of cytoadherence in P. vivax infections.
Funding: Funded by Generalitat de Catalunya, Ministerio Español de Economía y Competitividad, REDiEX, and Fundación Ramón Areces. HT is recipient of an AGAUR PhD fellowship

OF18.06

Oxidative stress alert by extracellular vesicles, in vitro study in ocular drainage system

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Introduction: The ocular drainage system is chronically exposed to oxidative stress (OS) contributing to cataract and primary open angle glaucoma (POAG) development. Classical markers of OS were found in patients ocular drainage tissues. The ability of EVs to deliver OS alert messages between the aqueous humor producing cells named non pigmented ciliary epithelium (NPCE) end the Trabecular Meshwork (TM) cells draining the aqueous humor was studied.

Methods: NPCE cells were exposed to OS and their released EVs were collected (Ox-EV). Non-stressed NPCE derived EVs (N-EV) were used as control. TM cells exposed to the same OS were treated with Ox-EV or N-EV and non-stressed TM cells were use as control. The EV treatment effect was measured by Nrf2-Keap1 signaling pathway changes including Nrf2 expression, related antioxidant gene expression, SOD and Catalase activity and TM cell antioxidant capacity.

Results: TM cells exposed to OS caused a significant 25% reduction in viability. When treated with Ox-EV the viability decrease was abolished. This cell rescue effect was not shown with N-EV treatment. Increase in Nrf2 cytosolic and nucleic expression was found following TM oxidative stress. Some nucleic but not cytosolic increase was found when N-EV treatment was done. The most pronounced significant increase cytosolic and nucleic expression was found following Ox-EV treatment. Antioxidant gene expression showed a significant increase following Ox-EV treatment in SOD2, GPx, HOX1 and NRF2 an effect that was not archived following N-EV treatment. SOD1 gene expression decreased following N-EV treatment but did not change when Ox-EV were used. Using the DCF-DA analytical method for total antioxidant capacity of TM cells we found that OX-EV treatment resulted in significantly higher antioxidant capacity vs N-EV or untreated TM cells. The two major antioxidant enzymes, SOD and Catalase activity was significantly higher following OX-EV treatment.

Summary/Conclusion: EVs are capable of OS alert to other cell resulting in a better antioxidant capacity. This phenomenon is relevant probably for all cells, can be the result of EVs cargo modification under OS including proteins and miRNAs or/and oxidized proteins, lipid and nucleic acids carried by the EVs as cargo or on their surface.

Funding: ISRAEL SCIENCE FOUNDATION (grant No. 1315/ 14).

PF01: EVs Immune System
Friday Poster Session
Chairs: Wilfrid Boireau; Saara Laitinen
Location: Level 3, Hall A

15:30–16:30

PF01.01

From adults to centenarians: characterization of T cell immunosenescence markers on plasma extracellular vesicles and their influence on T cell activation, viability and interleukin secretion

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Introduction: Aging is a universal, complex and heterogeneous process that leads to reduced adaptation capacity and increased vulnerability. Two of the hallmarks of aging are cellular senescence and altered intercellular communication. Specifically, the dysfunction and accumulation of senescent cells of the immune system is called immunosenescence. Regarding intercellular communication, the term senescence-associated secretory phenotype (SASP) is used and while inflammaging has been broadly studied, the role of extracellular vesicles (EVs) remains unclear. In the present work, we investigated the senescent features of plasma EVs and their role in T cell activation, viability and interleukin (IL) secretion.

Methods: All participants (24–104 years) gave informed consent and the study was approved by the Donostia University Hospital Ethics Committee. PBMCs were isolated with Ficoll-Hypaque method and EVs by differential centrifugation as described before by our group (Saenz-Cuesta et al., 2015). T cells were characterized by flow cytometry (FC) (FACSCanto II). Isolated EVs were detected by cryoEM, NTA and FC. The immunosenescence markers of EVs were also assessed by FC (CytoFLEX). Coculture experiments of PBMCs and EVs were performed and activation of T cells was induced by PHA. Cultured cells were evaluated by FC and the supernatants by Luminex for IL measurement.

Results: Senescent T cells accumulate with age, and CD8 cells are more affected than CD4 cells. Most of isolated EVs are 100–200 nm. They carry characteristic EV markers (CD63, CD81, CD9) as well as T cell markers, but no accumulation of EVs with senescent features was found with age. The co-culture of plasma EVs with PBMCs influences diverse aspects of T cells. They enhance cell viability. Besides, under PHA stimulation, EVs influence T cell activation and interleukin secretion. Interestingly, the effect of plasma EVs is distinct depending on the age of the donor.

Summary/Conclusion: Plasma EVs bear characteristic tetraspanins and T cell markers and can be detected by FC. However, EVs with senescent features do not accumulate with age. Moreover, EVs can influence T cell viability activation and IL secretion *in vitro*.

Funding: AA, IOQ and LI are supported by a fellowship from the Dept. of Education of the Basque Government

PF01.02

Profiling tetraspanin expression patterns on the surface of retroviruses and extracellular vesicles by nanoscale flow cytometry

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Introduction: Tetraspanins are membrane-associated proteins with variable functions. Given that extracellular vesicles (EVs) bud from the membranous structures of the cell, whether it be at the cell surface or through the endosome, they serendipitously uptake these membrane-spanning proteins, most notably CD9, CD63 and CD81. Retroviruses share numerous biophysical and biochemical features with EVs, and especially small EVs in the 80 – 150nm size range. They also have a membrane-derived envelope that captures tetraspanins, as they too can egress from the cell surface and from the endosomal pathway. Here we attempt to distinguish EVs from retroviruses according to their surface tetraspanin expression profiles.

Methods: Using nanoscale flow cytometry (NFC), we assessed the expression of tetraspanins CD9, CD63 and CD81 by optimized antibody labelling on different fluorescent retroviruses including murine leukemia virus (MLV), avian leukosis virus (ALV), and the

human immunodeficiency virus-1 (HIV-1). These retroviruses were engineered to express either eGFP or superfolder GFP (sfGFP) on their surface as a fusion protein with the viral envelope glycoprotein. We also analysed endogenous retroviruses released from murine T cell lines EL-4 and BW5147, and from primary splenocytes from various mouse strains.

Results: Differential levels of CD9, CD63 and CD81 expression was observed among the retrovirus strains. Also, the type of fluorescent reporter present influenced tetraspanin capture on the surface of these viruses. Overall, these expression patterns appear dissimilar to those present on EVs released from uninfected cells. Analysis of murine T cells lines and primary splenocytes also exhibit release of endogenous retroviruses, which was confirmed by Western Blot analysis of the p30 capsid protein.

Summary/Conclusion: Taken together, these findings suggest that caution must be exercised in the use of tetraspanins as identifying markers for EVs as they are also highly expressed on retroviruses, which are known to be prevalent in most animal species including birds, mice and humans. Furthermore, mouse studies should control for the presence of endogenous retroviruses, which may contaminate EV sample preparations.

Funding: Natural Sciences and Engineering Research Council of Canada (NSERC)

PF01.03

Isolation of EVs derived from human oral keratinocytes

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Introduction: Oral keratinocytes are the first defense line against external environments such as chemical agents, microbes and physical factors. Stimulated oral keratinocytes produce cytokines/chemokines to modulate local inflammatory status. Based on recent researches, not only cytokines/chemokines but extracellular vesicles (EVs) also regulate immune response. Therefore, we hypothesized that oral keratinocytes release EVs and those EVs could modulate immune response in the gingival tissue.

Methods: EVs were isolated from human oral keratinocytes (HOK-16B) by ultracentrifugation (UC) and commercial EVs isolation kit and analysed by western blotting and Nanoparticle Tracking Analysis (NTA).

Results: To exclude EVs originated from cell culture medium, we compared three different keratinocyte culture media, then we chose medium that contained the smallest number of particles. Two million HOK-16B cells released 10 billion EVs for 24 h. The EVs expressed general EV markers such as CD9, CD63, Flotillin-1 and Alix. According to the NTA the EVs were heterogeneous in size.

Summary/Conclusion: HOK-16B cells released EVs that have general EV markers. The EVs derived from HOK-16B infected with periodontopathogen need to analyse and confirm the biological function to other cells.

Funding: This work was supported by National Research Foundation of Korea grants (No. NRF-2018R1A5A2 024418 and NRF-2018R1A2A2A05018558).

PF01.04

Air pollution effects on the clinical course of autoimmune diseases: the role of extracellular vesicles

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Introduction: Autoimmune diseases (ADs) are characterized by the body's intolerance to self-antigens. The cause of autoimmunity is still unknown. However, it is generally accepted that ADs might be triggered by environmental factors able to increase inflammation. In recent years, extracellular vescicles (EVs) have been described to play an important role both in ADs pathogenesis and environmental toxicants, such as particulate matter (PM). The aim of our study is to evaluate PM effects on EV release in ADs.

Methods: We recruited 24 patients with ADs (12 Rheumathoid Arthritis, RA and 12 Systemic Sclerosis, SSc) and 12 patients with Osteoarthritis (OA), a non-autoimmune inflammatory disease taken as control. Plasma EVs were analysed by Nanosight and flow cytometry after labelling with the following markers: CD14+ (monocyte), CD61+ (platelet), CD25+ (T-reg), ERVWE1+ (human endogenous retrovirus W), HLAG + (human leukocyte antigen G). PM10 and PM2.5 concentrations at the residency of each subject were obtained from the regional air quality monitoring network.

Results: The increase of PM2.5 led to a decrease of MVs CD14+ ($\beta = -0.13$; $p < 0.01$) and CD61+ ($\beta = -0.08$; $p = 0.05$) in RA, of ERVWE1+ in both SSc ($\beta = -0.10$; $p = 0.01$) and OA ($\beta = -0.09$; p = 0.01), and of HLA+ ($\beta = -0.12$; $p < 0.01$) only in SSc. Similar results were observed analyzing PM10 exposure. Analysis of EVs concentration according to their

dimensions showed a negative association in the size range of exosomes (63–92 nm) in RA and SSc compared to OA ($p < 0.05$). Finally, we observed a negative association between exosomes and C-reactive protein (β = −1.99; p = 0,03), and a positive association between ERVWE1+ and Erythrocyte Sedimentation Rate (ESR) (β = 0.53; p = 0,06) and HLA+ and ESR (β = 0.29; p = 0,01).

Summary/Conclusion: Our findings showed that PM exposure could be a further risk factor of autoimmunity through a modulation of EV release.

PF01.05

The immunomodulatory effects of human umbilical cord perivascular cell-derived extracellular vesicles on T lymphocyte differentiation

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Introduction: We have characterized human umbilical cord perivascular cells (HUCPVC) as a promising source of mesenchymal stromal cells (MSC). Our previous data from *in vitro* and *in vivo* models of myocardial infarction and neurovascular injury support that HUCPVCs have potent immunomodulatory property, and in many cases, are superior to bone marrow MSCs. The immunomodulatory effects of HUCPVCs are thought to be contributed by paracrine factors. However, the role of HUCPVCs in immunomodulation is still unknown. Here, we reveal the immunomodulatory effects of HUCPVC-derived extracellular vesicles (EV) on T cell differentiation *in vitro*.

Methods: Conditioned medium (CM) was obtained from sub-confluent first trimester (FTM) and term HUCPVCs cultured for 48 hrs in serum-free RPMI medium with or without cytokines (10 ng/mL of IFN-γ, 15 ng/mL of TNF-α). HUCPVC-derived EVs were enriched from CM using the Qiagen exoEasy Maxi kit, followed by a Vivaspin 100k MWCO buffer exchange. Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient with written informed consent from healthy donors. PBMCs stimulated with anti-CD3/CD28 beads were co-culture with HUCPVCs or their EVs for five days. T cell differentiation and proliferation were analyzed by flow cytometry.

Results: HUCPVCs elicited an immunomodulatory effect by increasing the T regulatory cells/T effector cell (Treg/Teff) ratio in a paracrine manner, which could be partially impaired by the endosomal pathway inhibitor, GW4869. In the CD4+ population, HUCPVC-derived EVs promoted both the proliferation of Treg and Teff. Notably, the ratio of proliferating Treg/proliferating Teff is increased by HUCPVC-derived EVs treatment when compared to no cell-CM control isolation, which eventually resulted in an increase of Treg/Teff ratio. In the CD8+ population, administration of HUCPVC-derived EVs significantly shifted the CD8+ population towards a CD8low population. We found no significant difference in the effect of EVs derived from inflammatory primed and unprimed HUCPVCs.

Summary/Conclusion: HUCPVC-derived EVs demonstrated immunomodulatory effects by increasing Treg/Teff ratio in the CD4 T helper cells and shifting the cytotoxic T cell phenotype towards CD8low. We suggest that HUCPVC-derived EVs represent a promising cell-free immunomodulatory therapy.

PF01.06

Cytokine and miRNA profiling of plasma extracellular vesicles in individuals with myalgic encephalomyelitis/chronic fatigue syndrome

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Introduction: Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating disease of unknown aetiology lasting for 6 months or more, with features including fatigue, cognitive impairment, myalgias, postexertional malaise, and immune system dysfunction. Given the possibility that abnormal protein signaling could be altering cellular function in ME/CFS, we aimed to characterize the cytokine/chemokine profile and miRNA content of blood-derived Extracellular Vesicles (EVs) in ME/CFS individuals and healthy controls.

Methods: We included 35 ME/CFS patients and 35 sedentary controls matched for age, sex and BMI. EVs were enriched from plasma by precipitation and characterized by NTA, TEM and western blotting. A 43-plex immunoassay was used to determine cytokine/chemokine concentrations in both plasma and isolated EVs. Total RNA was isolated from EVs and small RNA libraries were prepared and sequenced for miRNA profiling.

Results: ME/CFS patients had significantly higher levels of EVs than controls and morphological analysis

showed a homogeneous population of vesicles in both groups. Comparison of cytokine concentrations in plasma and isolated EV for cases and controls yielded few significant results. Cases had higher plasma levels of MCP-1 and IP-10 and lower levels of RANTES, EGF and GROβ. In isolated EVs, EGF and GROβ were also significantly lower in patients while concentrations of IL-12p70, IL-2 and GM-CSF were higher. Correlations analysis revealed that CD40L was a negative driver of TRAIL and IP-10 in patients but had no inverse associations with other cytokines within the control group. We also generated on average over 800,000 miRNA-mapped reads per EV sample. A total of 316 mature miRNAs sequences were detected and no significant differences between groups after multiple corrections were found. PCA analysis of all ME/CFS and control miRNAs revealed a separation of the groups based on PC4.

Summary/Conclusion: Higher levels of pro-inflammatory cytokines were found in plasma and EVs from ME/CFS patients and inter-cytokine correlations revealed unusual regulatory relationships among cytokines in the ME/CFS group that were not apparent in the control group. Studies with expanded cohorts are needed to increase power of analysis.

Funding: This project was funded by NIH NIAID R01 AI107762.

PF01.08

Immunomodulatory exosomal signalling mediated by porous templated scaffolds

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Introduction: Porous templated scaffolds (PTS) with pores 40 μm in diameter drive healing upon implantation by reducing inflammation and foreign body rejection while increasing local angiogenesis. Macrophage recruitment and polarization are known to play roles in this phenomenon, but the mechanism driving this healing response is poorly understood. We believe 40 μm PTS resident immune cells are releasing exosomes containing unique cargo that modulates healing by influencing $CD4^+$ T cell subsets.

Methods: We quantified the cellular origin and internal composition of exosomes isolated from explanted 40 μm and 100 μm PTS using a Cre-Lox double transgenic mouse model and qPCR, respectively. We then quantified the cellular response to these exosomes *in vitro* using qPCR, ELISA and cell proliferation assays.

Results: Our evidence shows that immortalized T cells treated with exosomes isolated from explanted 40 μm PTS significantly down regulate TNF-α expression without other transcriptomic effects. Moreover, primary co-stimulated (CD3/CD28) T cells treated with exosomes from macrophages (MØ) resident to 100 μm PTS exhibited broad transcriptional upregulation and significantly upregulated proportional expression of several pro-inflammatory genes: TNFα, TBX21, GATA3 and TGF-β1. When treated with exosomes from MØ in 40 μm PTS, these T cells exhibited no broad transcriptional upregulation but proportionally upregulated immunomodulatory T cell phenotypic markers FoxP3 and IL-10.

Summary/Conclusion: These data show that exosomal signalling of PTS resident cells is controlled by pore size, thus influencing T cell differentiation and host response. Particularly, exosomes from cells in 100 μm PTS proportionally upregulate T cell markers associated with Th1, Th2 and Th3 T cell subpopulations and transcriptomic stimulation, whereas exosomes from 40 μm PTS induce a proportional upregulation of T cell markers associated with immunomodulatory Tregs, without broad transcriptomic stimulation. Our next experiments will examine the ability of exosomes generated in 40 μm PTS to recapitulate a healing response in implants known to otherwise promote the foreign body response.

PF01.09

Extracellular vesicles in systemic sclerosis as potential mediator for pulmonary vascular disease

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Introduction: Pulmonary vascular disease (PVD) is characterized by media muscular hypertrophy/hyperplasia. Recently, the deregulation of EVs in some forms of pulmonary hypertension studies has been reported, but data on pulmonary vascular disease are still lacking. We investigated whether EVs from SSc patients with or without established PVD can induce hypertrophy and/or hyperplasia in *in vitro* smooth muscle cells and to study vesicular miRNAs expression.

Methods: We isolated plasma EVs from: 3 SSc-PAH patients with established PVD under target therapy [PH+]; 3 SSc patients with high clinical risk without PVD [PH−]; 3 early SSc patients with low clinical risk

[Ea]; and 3 healthy control subjects. Smooth muscle cells were cultured in RPMI complete medium enriched with EVs purified from each study subject. Real-time cell growth was analysed with xCELLigence RTCA. miRNAs from both plasma and medium cell EVs were characterized and target prediction was performed via Diana Tools mirPath 2.0.

Results: Real-time analysis of cellular growth showed a brisker growth in every aliquot exposed to EVs with respect to the control. The intergroup comparison showed that EVs from controls induced an inferior growth in terms of cell index and doubling time. PH− showed the greatest effect on cell growth with respect to Ea and PH+ treated subjects. The most deregulated miRNA was miR-324-3p which was strongly downregulated in PH−, weakly downregulated in PH+ and upregulated in Ea. Bioinformatics prediction for 324-3p showed it to target lipids synthesis and metabolism pathways.

Summary/Conclusion: These results provide evidence that EV content may predispose to PVD. The observed miRNA is potentially linked with the effect on cellular growth, suggestive of a role in subjects with high risk to develop PVD. The potential implication of deregulated miRNAs, especially 324-3p, on lipids metabolism indicates that this pathway could be involved in the pathogenesis of SSc-PVD.

PF01.10

Extracellular vesicles-associated cytokines in human pathologies

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Introduction: Many human diseases progress due to low-level chronic immune activation associated with the release of cytokines. Recently, we found that many cytokines are released in association with extracellular vesicles (EVs) rather than in the soluble form. Here, we investigated these EV-associated cytokines in three human diseases that are known to be facilitated by improper immune activation.

Methods: Multiplexed bead-based assays of 33 free and EV-associated cytokines in culture supernatants, platelet poor plasma and amniotic fluid.

Results: HIV disease: Infection of human lymphoid tissue by HIV-1LAI.04 resulted in the increased release of cytokines of which IL-7, IL-21, IFN-, MIP-1 and RANTES were selectively increased in EV-associated form. After 13 days of tissue treatment with combined anti-retroviral therapy (ART) cytokines, in particular, those that were associated with EVs, remain upregulated in spite of complete suppression of HIV replication. Cardiovascular disease: Myocardial infarction (STEMI) is associated with increased production of various cytokines of which IL-2, IL-6, IL-18, Gro-α and MIG were selectively increased in EV-associated form. Logistic regression analysis of a cohort of 110 individuals demonstrated that healthy controls and STEMI patients can be discriminated solely based on the analysis of EV-associated cytokines with 82.3% sensitivity and 85.4% specificity. Pregnancy complications: Amniotic fluid from pregnancies with intra-amniotic infection (IAI) demonstrated significantly increased concentrations of almost all cytokines in EV-associated form, as well as an increase of cytokine fraction released in EV-associated form.

Summary/Conclusion: The increase of EV-associated cytokines is a common denominator for diverse human diseases associated with chronic immune activation. For three diverse human pathologies, we observed increased cytokines packaging in EVs. In HIV infection EV-associated cytokines failed to return to baseline with ART, increases in STEMI allow for discrimination from controls, and IAI increases EV-associated cytokines. EV-associated cytokines may contribute to various disease progressions and can be developed into diagnostic tools.

Funding: NIH/NICHD Intramural Program.

PF01.11

Isolation and characterization of serum exosomes from Cystic Fibrosis patients receiving lung transplant

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Introduction: Lung transplantation is an important therapeutic option for Cystic fibrosis (CF) patients with end-stage lung diseases. Long-term survival has been impaired by various post-transplant complications. There is a critical need for development of methods to noninvasively monitor and evaluate patient recovery. Exosomes are the key mediator of intercellular communication, regulator of immune system and have been considered a promising candidate for liquid biopsy. Here, we developed imaging flow cytometry method that enables easy quantification and characterization of the exosomes surface markers in clinical samples. We profiled circulating exosome in serum isolated from a cohort of CF lung transplant patients with two macrophage and T cell-associated panels.

Methods: Serum samples were acquired from 30 CF patients 0, 1, 3, 6 and 12 months following lung transplantation. Upon isolation, exosomes were labelled with CFSE and a macrophage (CD68, CD86, CD163) or T cell panel (CD3, CD4, CD8, CD45RO). Flow cytometry analysis was carried out with Amnis Imagestream X Mark II. Results were analysed with patients' clinical indicators including pulmonary function tests (PFTs) and chronic lung allograft dysfunction (CLAD) diagnosis.
Results: Using optimized staining protocol, exosomes isolated from a wide range of patient samples were labelled consistently. All seven selected surface markers were detected on circulating exosomes. Results indicate that markers were selectively expressed on exosomes and do not follow the classic immune cell subsets. Results indicated that pan markers (CD68, CD3) expressions were lower than some subset markers, especially pro-inflammatory CD86. Patients diagnosed with CLAD showed higher percentage of macrophage surface markers consistently than the ones without CLAD. Additionally, patients not diagnosed with CLAD demonstrated a higher percentage of CD45RO expressing exosomes than those with CLAD at 6 months.
Summary/Conclusion: Detection and profiling of macrophage and T cell associated surface markers on circulating exosomes provide additional insights into patients' recovery and immune processes. Imaging flow cytometry-based exosome profiling represents a novel and promising strategy in CF patients post-transplant follow-up.
Funding: 265-2368 LTR Training Funds Mulligan (PI) 09/01/2010-12/1/2025.

PF01.12

Loss of TP53 modifies the quantity and protein load of extracellular vesicles in leukemic B-cells

Elena Izquierdo[a], Daniela Vorholt[b], Janica Wiederstein[c], Liudmila Lobastova[b], Stuart Blakemore[b], Marcus Krüger[c], Hinrich Peter Hasen[b], Michael Hallek[d] and Christian Pallasch[b]

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Introduction: Macrophages are key effector cells of the chemo-immunotherapy (CIT) response in B-cell malignances. We have previously shown that loss of *TP53* in leukemic B-cells diminishes the anti-tumour capacity of macrophages upon CIT. Here, we investigated the potential p53-dependent mechanisms in leukemic B-cells that could alter the phagocytic capacity of macrophages upon CIT.
Methods: The proteomic profile of control and TP53-deficient leukemic B-cells, untreated or treated with mafosfamide, was analysed by mass spectrometry. EVs were isolated from control and TP53-deficient leukemic B cells by differential ultracentrifugation and their proteomic content was evaluated by mass spectrometry. Validation of protein expression was performed by Western Blot and flow cytometry. The measurements of exosomes concentration and size distribution were performed by NanoSight NS300 and ZetaView.
Results: 244 of 5785 proteins were observed to be significantly different between TP53-deficient and control leukemic B-cells, with 159 independent of mafosfamide treatment, 147 associated to mafosfamide and 86 modifications shared between DMSO and mafosfamide treatment. Enrichment analysis for GO terms showed that TP53-deficient leukemic B-cells exhibited mainly altered expression of proteins associated with EVs. We confirmed that TP53-deficient leukemic B-cells produced higher concentration of EVs and that the EV-protein content differed from control leukemic B-cells. Notably, 1239 of 2663 proteins were significantly different between TP53-deficient and control leukemic B-cells, 68 were exclusively detected in the control-derived EVs and 128 proteins were only found in the TP53-deficient-related EVs
Summary/Conclusion: The loss of TP53 drastically modifies the proteomic profile of leukemic B-cells and influences the protein expression of leukemic B-cells upon mafosfamide treatment. Especially, the loss of TP53 regulates the EV-related protein expression and EV production in leukemic B-cells

PF02: EVS in the Central and Peripheral Nervous System
Chairs: Sowmya Yelamanchili; Elena Batrakova
Location: Level 3, Hall A

15:30–16:30

PF02.01

The effect of exosome purification method on the detection of amyloid β in exosomes with Photooxidation-Induced Fluorescence Amplification (PIFA)

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Introduction: Blood-based diagnosis of disease using exosomes sometimes demands a highly sensitive bioassay to detect rare protein biomarkers. New assay methods were suggested to overcome the limitations of a conventional ELISA system such as digital ELISA or plasmonic ELISA. However, these methods need a special expensive equipment with the long process. We have developed a photo-oxidation-induced fluorescence amplification (PIFA) that can measure less than 1 pg/mL by continuous irradiation on resorufin for the photooxidation of chemi-fluorescent substrate amplex red. This paper demonstrated it can identify Alzheimer's disease (AD) patient from normal control (NC) by measuring a low level of amyloid beta(Aβ) in the neuronal exosome from plasma samples.
Methods: The level of resorufin was measured by PIFA to compare with conventional ELISA. The oligomer Aβ was detected by same antibody system whose capture antibody is same as detection antibody to exclude the signals from monomer Aβ. We isolated exosomes from plasma samples (AD:4, NC:4) by three methods: ultracentrifuge(UC), CD9 antibody-coated magnetic beads (MB) and ExoQuick with agarose precipitation (EQ). Exosomes were lysed with RIPA buffer and Aβ as a cargo protein in exosomes were measured by PIFA. ELISA was performed by an automated machine using polypropylene tip. After removing the tip with HRP-tagged detection antibody, the fluorescence was measured continuously to amplify the fluorescence.
Results: The LOD of PIFA in measuring oligomer Aβ was less than 100 fg/mL that was lower than 2 orders of magnitude than commercialized ELISA kit. The dynamic range of PIFA assay is more than 5 decades. The volume of plasma sample was 150 uL and the final volume of exosome was almost the same. The concentrations of UC and EQ are 8.16 × 10^10 and 5.77 × 10^10 particles/mL. The AUC (area under curve) in identifying AD was 1.0, 1.0, and 0.875 by UC, MB and EQ, respectively. The result showed it could clearly identify AD from NC.
Summary/Conclusion: Exosome isolations using the magnetic beads, the exosomes can be extracted even in a small amount of less than 50 μl. Therefore, it is advantageous that the sample is used less and the exosome can be isolated quickly. We believe that the reliability of human samples will be improved by an additional number of testing samples and optimization of PIFA assay.

PF02.02

Bioinformatic and biochemical evidence for extracellular vesicle remodelling in Huntington's disease

Francesca Farina[a], françois-Xavier Lejeune[b], Satish Sasidharan Nair[b], Frédéric Parmentier[b], Jessica Voisin[b], Lorena Martin-Jaular[c], Clotilde Thery[c] and Christian Neri[b]

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Introduction: Intercellular communication mediated by extracellular vesicles (EV) is emerging as a mechanism that is important to neuronal development and survival. Here, we investigated the features of EV signalling in response to Huntington's disease (HD), a neurodegenerative disease that is caused by CAG expansion in the Huntingtin gene and that shows a significant degree of clinical heterogeneity.
Methods: We applied an integrated approach in which we combined bioinformatic analysis of public HD datasets and biological analysis in cellular models of HD pathogenesis.
Results: Using network methods to integrate high-dimensional HD transcriptomic data, we built a computational model of the transition between different phases of the HD process: from cell differentiation (early phase) to dysfunctional striatum (intermediate phase) and finally advanced neurodegeneration (late phase). This model evidenced the deregulation of a set of genes associated with the biology of EVs from

the earliest to latest phases of the disease. To test this hypothesis experimentally, we analysed EVs in mouse and human neuronal cell models of HD pathogenesis. To this end, we analysed different EV subtypes, testing for changes in secreted level and protein cargo composition. The results suggest that EV subtypes, especially small EVs, possibly including exosomes, may be altered in these cells.

Summary/Conclusion: Collectively, these data point to EV remodelling in the course of HD. Biological and clinical implications will be discussed.

Funding: ANR, France

PF02.03

HIV-1 Tat-induced astrocytic extracellular vesicle miR-7 impairs synaptic architecture

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Introduction: Although combination antiretroviral therapy (cART) has improved the health of millions of those living with HIV, the penetration into the CNS of many such therapies is limited, thereby resulting in residual neurocognitive impairment, commonly referred to as NeuroHIV. Additionally, although cART can successfully suppress peripheral viremia, there is a continuous persistence of the cytotoxic viral Transactivator of transcription (Tat) protein in tissues such as the brain, thereby contributing to neuronal injury.

Methods: Transmission electron microscopy, NanoSight and western blot analyses were used to characterize astrocyte-derived EVs (ADEVs). Among the various dysregulated miRs in the ADEV cargo, miR-7 levels were found to be upregulated by real-time PCR. Uptake of ADEVs by neurons was assessed by confocal microscopy. Rodent hippocampal neurons were exposed to Tat-ADEVs and assessed for inhibitory (GAD65 and gephyrin) and excitatory (vGlut1 and PSD95) synapses by immunostaining and confocal microscopy.

Results: Expression level of miR-7 was upregulated in the astrocytes from SIV+/HIV+ brains. In addition, Tat-stimulated astrocytes also demonstrated upregulated expression and release of miR-7 in the EVs, that were taken up by neurons, resulting in synaptic injury. Furthermore, our results also demonstrated that exposure of hippocampal neurons to Tat-ADEVs resulted in decreased expression of neuronal NLGN2, which in turn, led to loss of both excitatory and inhibitory synaptic densities. Moreover, we also demonstrated a neuroprotective role of PDGF-CC in rescuing Tat-ADEV-mediated synaptic loss.

Summary/Conclusion: We demonstrate that exposure of astrocytes to HIV protein Tat mediates the induction and release of EV-miR-7 that is taken up by neurons, leading in turn, to downregulation of neuronal NLGN2 and ensuing synaptic alterations. Importantly, synaptic impairment could be reversed by pretreatment of neurons with a neurotropic factor PDGF-CC.

Funding: This work was supported by grants DA040397, MH112848 (S.B.) and DA042704, DA046831 (G.H.) from the National Institutes of Health. The support by Nebraska Center for Substance Abuse Research is acknowledged.

PF02.04

The pericytes-derived extracellular vesicle-mimetic nanovesicles rescues erectile function by enchancing penile neurovascular regeneration in a mouse model of cavernous nerve injury.

Jiyeon Ock[a], Guonan Yin[b], Mi-Hye Kwon[a], Kang-Moon Song[a], Kalyan Ghatak[a], Nguyen Nhat Minh[a], Min-Ji Choi[c], Yong Song Gho[d], Ji-Kan Ryu[a] and Jun-Kyu Suh[a]

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Introduction: Extracellular vesicles (EVs) contains various proteins, mRNA and miRNA, that have many regulatory effects on recipient cells. However, most mammalian cells release low quantities of EVs, therefore, we use bioengineered method and extract extracellular vesicle-mimetic nanovesicles from mouse cavernous pericyte. The aim of this study was to investigate effectiveness of pericytes-derived extracellular vesicle-mimetic nanovesicles (PC-NVs) in restoring erectile function in cavernous nerve injury (CNI) mice.

Methods: Twelve-week-old C57BL/6J mice were used and divided into into groups (N = 5 per group): sham operation group and bilateral cavernous nerve crushing group receiving a single intracavernous injection of PBS (20 μl) or PC-NVs (0.1 μg, 1 μg, 5 μg/20 μl, respectively). One week later, erectile function was measured by electrical stimulation of the cavernous nerve (N = 5 per group). The penis was then harvested and stained with antibodies to CD31, NG2 and βIII tubulin. We also determined the neurotrophic and angiogenic potential of PC-NVs in an *ex vivo* major pelvic ganglion (MPG) culture experiment.

Results: The PC-NVs was extract and characterized by several EVs markers, such as CD63, CD81 and TSG101. Intracavernous injections of PC-NVs significantly improved erectile function in CNI mice, which reached up to 84% of control values. PC-NVs induced significant

restoration of cavernous contents of endothelial cells, pericytes, and neuronal cells in CNI condition. Moreover, PC-NVs promoted major pelvic ganglion neurite sprouting in *ex vivo* culture experiment.

Summary/Conclusion: In light of critical role of neuropathy in the pathogenesis of radical prostatectomy-induced ED, pericytes-derived extracellular vesicle-mimetic nanovesicles successfully restored erectile function in cavernous nerve injury mice. Further studies are needed to clarify mechanism by which PC-NVs induces neuropathy repair.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (Guo Nan Yin, 2018R1C1B6003829).

PF02.05

Neuronal EVs and microglial activation in hypoxia

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Introduction: There is significant data suggesting that EVs play a key role in CNS injuries such as stroke. During these types of injury the penumbral tissue around the core of the injured area is hypoxic and inflammatory. Here, we aimed to test the hypothesis that hypoxia affects EV production in cells of the neurovascular unit.

Methods: Using endothelial cells, microglia and neurons, we subjected cells to short periods of oxygen and glucose deprivation (OGD) measuring both the EVs released into the medium, and the downstream effects of these EVs on cells.

Results: Brain-derived endothelial cells (b.END3) and microglia (BV-2) do not produce significant numbers of EVs in response to hypoxia. However, N2A neuronal cells do, both under normal and differentiated conditions. These EVs appear to be pro-inflammatory when applied to microglia in culture.

Summary/Conclusion: Together, these data suggest that EVs produce pro-inflammatory EVs during periods of hypoxia, capable of activating microglia. Characterizing these neuronal EVs more fully may enable us to determine whether they are also able to escape the CNS during brain injury and activate peripheral macrophage populations.

Funding: MRF University of Oxford, Rose Trees Trust

PF02.06

Comprehensive study of vesicular and non-vesicular-associated miRNAs from a volume of cerebrospinal fluid compatible with the clinical practice

Endika Prieto Fernández[a], Ana Maria Aransay[b], Felix Royo[c], Esperanza Gonzalez[c], Juan Jose Lozano[d], Borja Santos-Zorrozua[e], Nuria Macias-Camara[b], Monika Gonzalez[b], Raquel Perez Garay[f], Javier Benito[g], Africa Garcia-Orad[a] and Juan Manuel Falcon-Perez[c]

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Introduction: Cerebrospinal fluid (CSF) miRNAs have emerged as a potential low invasive diagnostic tool for central nervous system malignancies. However, they have not yet been implemented in the clinic since there is no a reliable and simple method established to analyse the limited amount of CSF obtained from patients, especially from infants.

Methods: We have compared six existing protocols for characterizing miRNAs from a clinically rational volume (i.e. 200 µl) of CSF. Four of the methods incorporated an extracellular vesicles (EVs) enrichment step and the other two aimed to extract miRNAs directly from cleared CSF. The efficiency of each method was assessed by real-time PCR (qPCR) and smallRNA sequencing (smallRNAseq). Additionally, by size-exclusion chromatography, we determined the distribution of miRNAs among different CSF components.

Results: We found that NOR and INV protocols were the most efficient. According to our results, NOR was very reproducible by qPCR, showed a good miRNA levels correlation between techniques, and presented a user-friendly protocol starting from low volumes of CSF. In addition, we identified a set of microRNAs enriched in CSF exosomes that are involved in neurodevelopmental pathways.

Summary/Conclusion: We found that different protocols purify specific miRNAs subpopulations and CSF exosomes isolated by size-exclusion chromatography contain miRNAs involved in neurodevelopment.

Funding: This work was supported by the Basque Government [IT989-16]; the Spanish Ministry of Economy and Competitiveness MINECO (SAF2015-66312), and the Ramon Areces Foundation (FRA-17-

JMF). We also thank MINECO for the REDIEX (Spanish Excellence Network in Exosomes) and the Severo Ochoa Excellence Accreditation (SEV-2016-0644).

PF02.07

Identifying plasma-derived extracellular vesicle (EV) contained biomarkers in the development of chronic neuropathic pain

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Introduction: Over 100 million Americans suffer from chronic pain. However, research into potentially novel biomarkers and therapeutics for chronic pain is lacking. microRNAs (miRNAs) are attractive candidates as biomarkers due to their conservation across species, stability in liquid biopsies, and variation that corresponds to a pathologic state. miRNAs can be sorted into extracellular vesicles (EVs) within the cell and released from the site of injury. EVs transfer cargo molecules between cells thus affecting key intercellular signalling pathways. The focus of this study was to determine the plasma-derived EV miRNA content in a chronic neuropathic pain model.

Methods: Male Sprague-Dawley rats underwent either spinal nerve ligation (SNL; $n = 6$) or sham ($n = 6$) surgery, while under anaesthesia. Mechanical allodynia (MA) was assessed and plasma-derived EV RNA was isolated at baseline and days 3 and 15 post-injury followed by small RNA sequencing. Differentially expressed (DE) miRNAs and gene target enrichment/signalling pathways analysis was performed using R packages and TargetScan/IPA, respectively.

Results: SNL rats displayed a time-dependent increase in MA from day 3 to day 15 post-injury. Our results indicate that 22 miRNAs at day 3, 74 miRNAs on day 15, and 33 miRNAs at both day 3 and 15 were uniquely differentially expressed between the SNL and sham groups. The plasma-derived DE EV miRNAs were also found to regulate processes important in the development and maintenance of neuropathic pain states.

Summary/Conclusion: The key findings from this proposal include (1) the majority of the DE EV miRNAs, which normally function to suppress inflammation, were downregulated, and (2) twenty-one of the plasma-derived DE EV miRNAs reflect previously observed changes in the injured L5 nerve. This suggests that the plasma-derived EVs can potentially serve as key regulators, biomarkers and targets in the progression and treatment of neuropathic pain.

Funding: This research was supported by Congressionally Directed Medical Research Programs-Applied Pain Research (MR157005C). Research was conducted in compliance with the Animal Welfare Act, and all other Federal requirements. The views expressed are those of the authors and do not constitute endorsement by the U. S. Army.

PF02.08

A pilot study to evaluate serum miRNA from extracellular vesicles of neural origin for insight into neurological disorders

Michelle M. Barnett[a], Michael Geaghan[a], Ebrahim Mahmoudi[a], Judith Weidenhofer[b] and Murray Cairns[c]

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Introduction: miRNA are important regulators of gene expression and can be informative biomarkers of disease state. Alteration of miRNA expression in the brain has been observed in several neurological and psychiatric disorders postmortem, but they are almost impossible to non-invasively biopsy. These molecules, however, have been observed in serum and serum vesicles and have application as trait biomarkers. While it is possible that brain disorders also have systemic changes that contribute to the serum profile it is reasonable to suspect that extracellular vesicles (EV) that originated in the brain would have more specificity and relevance to the neuropathology. In this pilot study we investigated strategies for capturing and profiling the miRNA content from extracellular vesicles derived from the brain using an immunoaffinity approach.

Methods: Whole blood from a healthy male volunteer was collected in serum separator tubes, immediately processed and stored at −80°C. Antibody-coupled magnetic beads were incubated with serum and cell culture EV. Enriched and non-enriched fractions were recovered using a commercial spin column kit for EV purification and RNA extraction. Small RNA libraries were prepared for 75 cycles of single end multiplex sequencing. Raw reads were converted to fastq, adaptors trimmed, reads mapped, aligned and counted.

Results: miRNA were identified in both total human serum and the neural fractions. Functional annotation of the 37 miRNA present in the neural EV revealed that neuron projection was the most enriched cellular

component in predicted targets, suggesting that they may serve to augment the synaptic regulatory environment. In support of this hypothesis, we found over representation of all four cytoplasmic polyadenylation element binding proteins (CPEB1-4) among targets predicted to be regulated by the most abundant miRNA. CPEB proteins affect translation of mRNA bearing polyadenylation element sequences and alterations in the expression of these proteins have been associated with synaptic plasticity, intellectual disability and autism spectrum disorder.

Summary/Conclusion: Serum EV can be enriched for those of neuronal origin and this strategy may provide insight into the brain's regulatory environment and ultimately more sensitive biomarkers of neurological and psychiatric disorders.

PF03: EVs Cancer Metastasis
Chairs: Ryou-u Takahashi; Irina Nazarenko
Location: Level 3, Hall A

15:30–16:30

PF03.01

Promotion of metastasis via alteration of vascular endothelium by tumour exosome miRNA

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Introduction: We have reported that tumour endothelial cells acquire diversity by cancer secreting factors. Cancer cells secrete exosomes to create a suitable environment for themselves. miRNA (miR) is transported by exosome from cell to cell. We have identified miR-1246 that is more abundant in high metastatic melanoma (A375SM) exosomes compared with in low metastatic melanoma (A375) exosomes by miRNA array analysis. In this study, we investigated the role of miR-1246 in alteration of the character of endothelial cells (ECs). In addition, we addressed the mechanism of cancer metastasis induced by miR-1246.
Methods: We focused on the adhesion between ECs and between cancer cells and ECs. Changes in adhesion molecule expression and endothelial permeability were examined. We analysed the effect of the administration of A375SM exosome and miR-1246 knockdown on lung metastasis *in vivo*. In addition, exosome miR-1246 levels in blood of melanoma and oral cancer patients were compared with healthy subjects.
Results: miR-1246 transfection in ECs increased the expression of adhesion molecule, ICAM-1 via activation of STAT3, and increased the number of adherent cancer cells to ECs. Furthremore, A375SM exosome treatment enhanced endothelial permeability. Also, the expression of VE-Cadherin, which is intracellular adhesion molecule of EC and function as EC barrier, was downregulated by miR-1246 transfection. The target gene prediction database and reporter assay showed that VE-Cadherin is the target gene of miR-1246. Intravenous administration of A375SM exosome-induced cancer cell colonization in the lung, resulting in establish of lung metastasis. In contrast, miR-1246 knockdown in A375SM caused in decrease of lung metastasis *in vivo* model. Exosome miR-1246 levels in blood of melanoma and oral cancer patients were significantly higher than those in healthy subjects.
Summary/Conclusion: Thus, it was suggested that miR-1246 in tumour-derived exosomes promotes lung metastasis by inducing the adhesion of cancer cells to ECs and destroying the EC barrier.

PF03.02

Patient-derived circulating exosomes enhance cancer and stemness properties through polymeric immunoglobulin receptor in liver cancer

Sze Keong Tey and Judy Yam

The University of Hong Kong, Hong Kong, Hong Kong

Introduction: Hepatocellular carcinoma (HCC) is a primary malignancy of liver which often diagnosed at an advanced stage accompanied by extrahepatic metastasis. Emerging evidences have demonstrated cancer cells derived exosomes play an important role in intercellular communication in tumour microenvironment during metastasis. Thus, exosome research may solve the mystery of metastatic organotropism in HCC.
Methods: Exosomes were isolated from HCC patients. The biological effects of exosomes were studied using transwell and matrigel invasion assays. The in vivo effect of exosomes were analysed in mice by intravenous injection of exosomes together with hepatoma cells. The *in vivo* lung pulmonary vasculature and vascular leakiness were revealed by FITC-lectin stain and presence of Texas Red-dextran, respectively. Stem-like properties were examined by spheroid assay and flow cytometry. Proteomic profiling and expression level of exosomal proteins were analysed by mass spectrometry and enzyme-linked immunosorbent assay (ELISA), respectively.
Results: We recruited representative individuals of different stages of HCC development for the collection of circulating exosomes, namely, exosomes of healthy individual (Normal-Exo), patient with cirrhosis (Cirrhosis-Exo), HCC patients at early stage (E-HCC-Exo) and late stage with distant metastasis to lungs (L-HCC-Exo). Among these exosomes, L-HCC-Exo exhibited the highest effect in promoting HCC cell

migration and invasion *in vitro* and colonization of hepatoma cells in lungs. L-HCC-Exo also modulated tumour microenvironment by enhancing pulmonary vascular permeability. Along with cancerous behaviours, L-HCC-Exo induced stem-like properties such as increased self-renewal, expressions of hepatic CSC markers and drug resistance. Elevated level and activity of β-catenin were also detected in cells stimulated by L-HCC-Exo. Proteomic profiling of exosomal proteins identified polymeric immunoglobulin receptor (pIgR) to be markedly upregulated in L-HCC-Exo when compared with exosomes of other subjects.

Summary/Conclusion: HCC patient-derived circulating exosomes augment cancer and stemness properties through the activation of β-catenin signalling by exo-pIgR, leading to a more aggressive phenotype in HCC tumourigenesis and metastasis.

PF03.03

Ectopic ATP synthase induces extracellular vesicle release for cell-to-cell communications

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Introduction: Ectopic Adenosine triphosphate synthase (eATP synthase) is defined as ATP synthase located on cell surface instead of inner membrane of mitochondria. Our previous studies showed eATP synthase located on lung cancer cell surface and generated ATP to extracellular space. Recently, several reports indicated that the subunits of ATP synthase were identified in extracellular vesicles (EVs) using proteomics approach. However, where does ATP synthase in EVs come from and what are the functions of it are still unclear. We proposed the hypothesis: ATP synthase in EVs may be conveyed from cell surface for cell-to-cell communications.

Methods: Here we used immunochemistry to detect eATP synthases and serial high-speed centrifugation to isolate EVs including apoptotic bodies, microvesicles and exosomes which were further confirmed by transmission electron microscopy (TEM) and nano tracking analysis (NTA). Furthermore, we used quantitative proteomics by dimethyl labelling to profile the proteomes in extracellular vesicles, and dot blot analysis to elucidate whether ATP synthase was localized on the EV membrane.

Results: We identified 892, 311 and 331 proteins including ATP synthase subunits in apoptotic bodies, microvesicles and exosomes, respectively. We further confirmed that ATP synthase was indeed localized on EV membrane. Additionally, we observed the release of these three subtypes of EVs was decreased after starvation stress and an eATP synthase inhibitor citreoviridin treatment. However, we did not measure the significantly different ATP production between control and citreoviridin treatment in apoptotic bodies, microvesicles and exosomes, indicating that ATP synthase on the EVs may not be for ATP synthesis. We observed that eATP synthase was transferred from cancer cells to normal cells via EVs, indicating eATP synthase plays an important role for cell-to-cell communications and eventually promotes cancer metastasis.

Summary/Conclusion: Our findings suggest that ATP synthase indeed exists on the membrane of EVs and enhances cancer cells to release EVs for cell-to-cell communications.

Funding: Ministry of Science and Technology (MOST 106-2320-B-002-053-MY3) and National Health Research Institutes. (NHRI-EX107-10709BI) in Taiwan.

PF03.04

Anesthesia-dependent changes in vesicular miRNA profiles during colorectal cancer surgery

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Introduction: Colorectal cancer (CRC) is one of the most common and most deadly cancer types worldwide, leading to 50,000 annual deaths in the US alone. Even though surgical resection presents the best chance of survival for CRC patients, accumulating evidence demonstrates that removal of primary tumours can foster disease progression and metastasis. Recent outcome-based studies described differential effects of the type of anaesthesia used during CRC surgery on metastasis as well as overall and recurrence-free survival. As mechanistic data on how anaesthesia impacts cancer progression are sparse, we assessed the potential involvement of extracellular vesicles (EVs) in the process.

Methods: Serum was sampled from 18 CRC resection patients before induction of anaesthesia (pre) using

propofol ($n = 8$) or sevoflurane ($n = 10$) and after surgery (post). EVs were precipitated from 1 ml serum, and associated microRNAs (miRNAs) were profiled by Next-Generation Sequencing. The anaesthesia-dependent impact on miRNA profiles in paired EV samples was assessed using DESeq2. Next, we performed pathway analyses based on differentially regulated miRNAs. Additionally, deregulated candidates selected from NGS data were validated by RT-qPCR.
Results: NGS-based profiling of EVs resulted in 3.79E6 ± 1.58E6 (propofol pre), 3.09E6 ± 1.81E6 (propofol post), 3.40E6 ± 1.65E6 (sevoflurane pre) and 3.34E6 ± 1.32E6 (sevoflurane post) mean miRNA reads per sample. As evidenced by Principal Component Analysis, samples from pre- and post-operative sera clustered into distinct groups for both types of anaesthesia. Differential expression analysis revealed 64 and 44 miRNAs significantly regulated by propofol and sevoflurane, respectively. Despite substantial overlap in the intraoperative miRNA changes, a set of 31 (propofol) and 11 (sevoflurane) miRNAs specifically responsive to either drug was also identified. *In silico* analyses indicated a differential effect of anaesthesia-responsive miRNAs on cancer-relevant pathways such as proliferation, apoptosis and migration.
Summary/Conclusion: Previous studies have demonstrated distinctive effects of propofol and sevoflurane on tumour cells, host immunity and survival in CRC. Anaesthesia-induced changes in circulating miRNAs might mediate disease progression and impact post-surgical outcome.

PF03.05

The role of hypoxia-derived exosomes in determining Neuroblastoma dissemination and aggressiveness

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Introduction: Neuroblastoma (NB) is a heterogeneous paediatric malignancy of the sympathetic nervous system accounting for up to 10% of childhood cancers with a strong tendency to metastasize. Hypoxia is a key feature of solid tumours and is specifically known to (i) favour NB metastasis and dedifferentiation towards immature stem cell-like phenotypes and to (ii) stimulate release of exosomes (EXO), facilitating intercellular communication at distant sites. In this study, we characterized the proteomic and miRNAs cargo of EXO isolated from NB cell lines cultured at different oxygen concentrations to identify an exosomal signature associated with NB metastatic dissemination.
Methods: SKNAS and SKNDZ NB cell lines were cultured for 48 h in standard (20% O2) and hypoxic (1.5% O2) conditions. EXO were purified from the media using Ultra spin tubes 100K MWCO and characterized by scanning electron microscopy (SEM) and qNANO. Proteome and miRNA cargo profiles were analysed by quantitative mass spectrometry and FirePlex Discovery Panel (on 405 miRNAs), respectively, and surface markers were evaluated using MACSplex technology.
Results: SEM and qNANO size distribution analysis gave populations of round particles within the expected diameters (50–120 nm). Surface markers analysis revealed that NB hypoxia-derived EXO express an increase of proteins associated with angiogenesis, adhesion, stemness and immune function such as CD105, CD29, CD49e, SSEA4, HLA-DR and HLA-ABC. We characterized the proteomic cargo of EXO isolated from cultures in normal and hypoxic conditions revealing differential expression of about 90 proteins. These preliminary results highlight relevant changes in the expression of several markers of EXO derived from cultures exposed to different oxygen concentrations.
Summary/Conclusion: We successfully isolated and purified exosomes from NB cell lines and assessed their protein composition. These promising results are the starting point for the identification of predictive biomarkers to be used to detect and monitor metastatic spread in NB.
Funding: ERC Starting Grant 2017 to Elisa Cimetta.

PF03.06

HNSCC exosomes drive tumour angiogenesis via ephrin reverse signalling

Shinya Sato and Alissa Weaver

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Introduction: Exosomes are small extracellular vesicles (EVs) that are secreted upon fusion of multivesicular endosomes (MVE) with the plasma membrane and carry bioactive protein and RNA cargoes. A number of studies have identified key roles for exosomes in promoting tumour angiogenesis; however, the mechanisms are unclear. Our goal is to identify the role of head and neck squamous cell carcinoma (HNSCC) exosomes in tumour angiogenesis.
Methods: EVs were collected from the conditioned media of HNSCCs and purified through cushioned

density gradient ultracentrifugation. An orthotopic mouse model was used for the assessment of tumour angiogenesis. Angiogenic potential of EVs was assessed by tube formation assays with Human Umbilical Vein Endothelial Cells (HUVECs).

Results: In HNSCC tumours, the microvessel density correlated with exosome secretion rates of original HNSCC lines. *In vitro*, CM and purified exosomes but not exosome-depleted CM from HNSCC cells drove tube formation of HUVECs and human lymphatic endothelial cells. Proteomics analysis of HNSCC exosomes revealed multiple potential angiogenic proteins, including EphB2 and EphB4. The addition of purified HNSCC exosomes to HUVECs-induced reverse ephrin-B signalling in endothelial cells, as assessed by Western blot analysis. To test whether reverse ephrin-B signalling might account for exosome-induced angiogenesis, we pre-incubated purified exosomes with Fc-ephrin-B2 to block the interaction between exosomal EphB2 and ephrin-B2 on endothelial cells. We found that low concentrations of this reagent had little effect on endothelial tube formation in the absence of exosomes but blocked the pro-angiogenic effect of the exosomes. In addition, EphB2-KD HNSCC derived exosomes significantly reduced endothelial tube formation compared to parental HNSCC derived exosomes.

Summary/Conclusion: We find that HNSCC-derived exosomes can induce reverse ephrin-B signalling and angiogenesis. This mechanism may be important in the HNSCC microenvironment.

Funding: This work was funded by the National Institutes of Health grant R01CA163592.

PF03.07

Nanoparticle mediated inhibition of intercellular communication between enzalutamide resistant prostate cancer cells and myeloid cells

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Introduction: Crosstalk between neoplastic cells and myeloid cells has emerged as an axis of communication which drives tumour progression and metastasis. Recently, our group and others have shown that cancer exosome-mediated intercellular signalling is dependent, in part, upon target cell cholesterol homeostasis. In this study, we investigated whether exosome signalling between enzalutamide resistant (EnzR) prostate cancer cells and myeloid cells could be effectively inhibited by targeted reduction of myeloid cell cholesterol using high density lipoprotein mimetic nanoparticles (HDL NPs).

Methods: Exosomes were isolated via ultracentrifugation of conditioned media from EnzR CWR-R1 prostate cancer cells. Murine bone marrow macrophages were obtained by culturing total bone marrow in M-CSF for 7 days. For *in vitro* experiments, cells were treated with exosomes derived from EnzR CWR-R1 cells (1–10 ug/mL exosomal protein) with or without HDL NPs (50–150 nM). For *in vivo* experiments, 10 ug exosomal protein were injected via tail vein with or without HDL NPs (1 uM, 100 ul). Confocal microscopy and flow cytometry were used for uptake experiments. Osteoclast differentiation assays were performed using a commercially available TRAP staining kit (Sigma Aldrich). NF-kB activation assays were performed using the human monocyte reporter cell line, THP-1 Dual. HDL NPs were synthesized using 5 nm gold nanoparticle templates, phospholipids, and apolipoprotein A-1. Mechanistic studies were performed using transgenic, SR-B1 knockout mice.

Results: Results showed that myeloid cell uptake of EnzR CWR-R1 exosomes was inhibited *in vitro* and *in vivo* upon treatment with HDL NPs. Furthermore, functional inhibition was observed via reduced osteoclast differentiation and reduced stimulation of NF-kB signalling. Finally, experiments conducted using SR-B1 knockout mice revealed that nanoparticle inhibition is dependent upon the scavenger receptor, SR-B1.

Summary/Conclusion: Our findings demonstrate that exosome-mediated signalling between prostate cancer cells and myeloid cells can be inhibited using HDL NPs. Furthermore, our results strongly suggest that exosome-mediated crosstalk between prostate cancer cells and myeloid cells are dependent upon cholesterol homeostasis.

Funding: This work was supported by the National Institutes of Health and the Prostate Cancer Foundation.

PF03.08

High-grade bladder cancer cells secrete extracellular vesicles containing MiRNA-146a-5p and promotes angiogenesis

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Introduction: High recurrence is one of the major issues in bladder cancer (BCa). The classical method for detecting recurrence is cystoscopy, a highly invasive technique. Thus, novel methodologies with high reliability and low-invasiveness are needed. To overcome this problem, biomarkers in the urine such as microRNA (miRNA) in extracellular vesicles (EVs) are been proposed. We previously detected high urinary levels of miR-146a-5p in patients with BCa related to tumour grade. However, the function of this miRNA in EVs from BCa had not been elucidated yet. Here, we show that miRNA-146a-5p in EVs promoted angiogenesis in BCa.
Methods: High-grade BCa cell line, UMUC-3, with miR-146a overexpression, was established and orthotopically transplanted in SCID mice. Immunohistochemical analysis was performed to evaluate angiogenesis. Cellular proliferation, migration, and invasion were assessed in human umbilical vein cell (HUVEC) after the addition of EVs from BCa. The target gene of miR146-5p was identified by microarray and *in silico* analyses, and downregulation was further confirmed by qPCR and western blot.
Results: Urinary miR-146a-5p level was higher in patients with high-grade BCa and the tumours presented high levels of angiogenesis. Similarly, miR-146a overexpressed BCa cells orthotopically injected into mice generated tumours with high levels of angiogenesis. HUVEC cell proliferation was significantly increased, both under transfection of miR-146a mimic and treatment with miR-146a-enriched EVs. Moreover, the target of miR-146a was found to be TET2, which has been reported to regulate cell growth in other malignancies. Consequently, TET2 was downregulated, both at RNA and protein level, under miRNA-146a-enriched EVs treatment.
Summary/Conclusion: Our findings indicate that EVs containing miR-146a-5p from BCa, previously described as BCa biomarker, promoted the proliferation of endothelial cells that supported tumour growth. These results demonstrate that miRNAs in EVs may become not only a diagnosis tool but also a target molecule for cancer therapy.

PF03.09

Activated glial cells stimulated by breast cancer-derived exosomes enhance proliferation of brain metastatic breast cancer cells

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Introduction: Brain metastatic breast cancer cells have been known to stimulate glial cells in the brain to facilitate their brain metastasis. Long distance of primary breast cancer site to the brain may require the communication mediator to deliver cancer favourable information to the brain. However, the exact role of breast cancer-derived exosome during brain metastasis is not well understood. In this study, we observed the phenomenon that breast cancer-derived exosomes directly activate primary astrocytes and the co-culture condition of these activated astrocytes with microglia cells enhances cancer cell proliferation and invasion.
Methods: To trace the extracellular vesicle (EV) including exosome movement, Palm-tandem dimer tdTomato (Palm-tdTomato) lentiviral vector was transduced into MDA-MB-luc-D3H2LN (D3H2LN) breast cancer cells. EVs isolated from D3H2LN-Palm-tdTomato cell lines showed the increased Palm-tdTomato fluorescence intensity and were stably internalized into astrocytes. After astrocytes were treated by the EVs, we checked the level of Glial Fibrillar Acidic Protein (GFAP), vimentin, MCP-1/CCL2 and IL-6 expression. Astrocytes and microglia are co-cultured under the EVs containing media.
Results: We found that astrocytes taken up by cancer-derived exosomes were activated, showing the increase in GFAP, vimentin, MCP-1/CCL2 and IL-6 expression. Also, we found that co-culture glial cells of astrocytes and microglia significantly increased cytokine IL-6 production. The co-cultured medium from cancer exosomes-stimulated astrocytes and microglia increases invasion and proliferation of cancer cells and inhibits tumour suppressor gene in breast cancer cells.
Summary/Conclusion: These results indicate that breast cancer-derived exosomes participate in activating astrocytes and the activated astrocytes and microglia induce breast cancer proliferation and invasion during brain metastasis.

PF03.10

The glycosylation status affects the biodistribution of cancer extracellular vesicles

Akiko Kogure[a], Nao Nishida-Aoki[b], Naoomi Tominaga[c], Nobuyoshi Kosaka[d] and Takahiro Ochiya[d]

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Introduction: It has been shown that extracellular vesicles (EVs) from cancer cells delivered to the metastatic site, and promoted metastasis by communicating with microenvironmental cells, although molecules, which are indispensable for cancer progression, has been investigating yet. It is well known that aberrant

glycosylation is a hallmark of cancer, and is related to the cancer malignancy; however, the role of the glycosylation of EV surface proteins in cancer progression has not been clarified yet. In this study, we investigated the role of glycosylation of the EVs from metastatic cancer cells in the biodistribution.

Methods: We performed lectin blot analysis in order to compare the glycan level of the EVs among metastatic cancer cell lines and non-metastatic cancer cell lines. Then, we investigated whether glycosylation of EVs affects their incorporation rate to endothelial cells by enzymatic deglycosylation *in vitro*. DiR-labelled EVs were employed to analyse the location of EVs *in vivo* by intravenous injection. After 24 h of injection, the fluorescence intensities of each organ were measured in order to determine the amount of the EVs remained at the organs.

Results: We found that the glycosylation level of EVs from metastatic cancer cells was higher than that from non-metastatic cancer cells. Moreover, enzymatic digestion of N- and O-linked glycans on EVs increased their incorporation to the endothelial cells *in vitro*. Furthermore, we found that the glycosylation status of EVs from cancer cells influenced their direction to the organs in mice.

Summary/Conclusion: These findings suggest that the glycosylation of EVs from cancer cells involved in the biodistribution of EVs.

PF04: EV-mediated inter-organism communication
Chairs: Chitose Oneyama; Kyoko Hida
Location: Level 3, Hall A

15:30–16:30

PF04.01

Preferential packaging of tRNA fragments into extracellular vesicles released by protozoan parasite Trichomonas vaginalis

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Introduction: Extracellular vesicles (EVs) are important mediators of cell-to-cell communication. Delivery of EVs is known to modulate the response of the recipient cells. EVs are produced by most if not all organisms and are involved in communication between host and pathogen. *Trichomonas vaginalis* is a unicellular eukaryotic pathogen, known to produce EVs with proteins and RNA cargo. This parasite colonizes the mucosal surface of the human genitourinary track extracellularly. In this study, we hypothesised that the RNA cargo of parasite EVs is an important element of this host-pathogen communication.
Methods: As the first step of this investigation, we isolated and characterised EVs from *T. vaginalis* strain B7RC2. Small RNAs present in these vesicles were identified by deep-sequencing and specificity of these molecules as EVs cargo was evaluated.
Results: Our results show that *T. vaginalis* releases membrane-bound vesicles with an average size of ~100 nm that are taken up by host cells. These vesicles are depleted of DNA but enriched with RNAs of small size. These RNAs are physically protected from exogenous RNases. The population of small RNAs was consistent among libraries, with tRNA being the most abundant RNA biotype in all samples. We identified individual sequences from the top 30 transcript clusters as being mostly tRNA fragments, particularly 5'-tRNA halves. The presence of the identified fragments was validated and compared with total cells by digital droplet PCR, showing the preferential packaging for these tRNAs into EVs.
Summary/Conclusion: Our study indicates that tRNA fragments from *T. vaginalis* EVs (particularly tRNA halves) might play a role in communication with host cells. Work to confirm their bioactivity continues.

PF04.02

The endothelial PlGF is upregulated by exosomes from activated kidney fibroblast

Noritoshi Kato[a], Fumitoshi Nishio[b], Yoshio Funahashi[c], Hiroki Kitai[c], Shintaro Komatsu[c] and Shoichi Maruyama[c]

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Introduction: It is well known that patients with chronic kidney disease (CKD) are at risk of cardiovascular diseases, but the mechanism of this distant organ crosstalk is not fully understood. Recently, placental growth factor (PlGF) received attention in pathogenesis of cardio-renal syndrome (CRS). Under the hypothesis that exosomes are involved in pathophysiology of CRS, the aim of this study is to explore the role of exosomes from kidney fibroblasts, which actively proliferate in diseased kidney, on vascular endothelial cells.
Methods: Clinical samples; HUVECs were stimulated by serum exosomes from stage G5 CKD patients and healthy donor. Exosomes tracking; Primary culture of activated kidney fibroblasts were obtained from experimental renal fibrosis model mice. These exosomes were labelled by microRNA of *C. elegance* (Cel-miR-39) then labelled exosomes were injected to the mice through tail vein. Effects of exosomes on endothelial cells; We purified exosomes from culture media of TGF-b stimulated kidney fibroblasts cell line (NRK-49f), and then primary culture of vascular endothelial cells (RAOEC) was stimulated using these exosomes. By qPCR, we evaluated the expression of PlGF genes.
Results: (1) Not only the serum but also exosomes from CKD stage G5 patients stimulated PlGF expression on HUVECs. (2) Injected labelled exosomes from activated kidney fibroblast distributed mainly in lung, liver and aorta. (3) RAOEC stimulated with exosomes form TGF-b activated rat kidney fibroblast showed higher expression of PlGF than control.
Summary/Conclusion: So far, CRS is considered to be caused by uremic factor, RAS system, chronic inflammation and so on. From this study, both serum and exosomes from CKD patients stimulated PlGF

transcription on endothelial cells. Exosomes from activated kidney fibroblast had same tendency. We speculated that exosomes from diseased kidney have some roles in atherosclerotic change by modulating the expression of PlGF on endothelial cells. Farther studies are needed to elucidate the degree of contribution to CRS.

Funding: N/A.

PF04.03

The effect of *in vivo* irradiation on the extracellular vesicle's cargo and uptake

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Introduction: Recent studies suggest that ionizing radiation (IR), as a stress agent, induces changes in the release, uptake and composition of extracellular vesicles (EVs). EVs were shown to play a role in radiation-related signalling and radiation induced bystander effects (RIBE). We have recently shown that EVs released by bone marrow (BM) cells of mice irradiated with X-rays mediate RIBE, such as DNA damages, chromosomal aberrations or phenotypical changes in certain cellular subpopulations of the BM. The aim of this study is to investigate the mechanism of these functional changes.

Methods: In order to follow the uptake of irradiated EVs, we isolated EVs from BM of total-body irradiated (TBI) mice, labelled them with a selective RNA stain and co-incubated them *in vitro* for 3 h with BM cells extracted from nonirradiated mice. We quantified the uptake of EVs in different BM subpopulations by flow cytometry and fluorescence microscopy. To test whether in vivo irradiation affects the miRNA cargo of EVs, total RNA was isolated from the same EVs, subjected to miRNA profiling and assessed by bioinformatical tools. Significantly altered miRNAs were validated by qRT-PCR in EVs, BM cells of EV donor and recipient mice.

Results: There were differences in EV uptake capacity of different BM cell subpopulations but irradiation did not change the extent of EV uptake. We identified a panel of miRNAs differentially expressed in the EVs following TBI of mice with involvement in DNA damage repair, immune system regulation and acute leukaemia.

Summary/Conclusion: We proved that EVs transmit certain radiation related signals; IR alters the miRNA cargo of EVs, but does not affect their uptake. Our study helps to disclose the radiation-related mechanisms involved in EV signalling and the role of EV signalling in systemic response of organisms to IR.

Funding: The Euratom research and training programme 2014–2018 (CONCERT, grant agreement number 662287) and a Hungarian Scientific Research Fund–OTKA (124879).

PF04.04

UVB induced-release of bioactive microvesicle particles in keratinocytes carry platelet-activating factor

Ji Bihl, Langni Liu, Christine Rapp and Jeffrey Travers

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Introduction: Ultraviolet B radiation (290–320 nm; UVB) has profound effects upon skin and generates systemic consequences. As UVB only penetrates the epidermis, a major question in photobiology is how UVB-treated skin sends systemic signals. Recent studies have indicated that small membrane-bound vesicles known as microvesicle particles (MVP) released from cells in response to various stressors can act as potent signalling agents due to their ability to carry nuclear and cytoplasmic components. Our lab has previously determined that UVB induces the production of the lipid mediator, platelet-activating factor (PAF), which is involved in mediating both acute pro-inflammatory and immunosuppressive UVB responses. More recently, we discovered that UVB generates MVP release (UVB-MVP) from epithelial cells and skin in a PAF-PAFR dependent way. However, the contents of UVB-MVP have not identified and whether UVB-MVP carry PAF is not known.

Methods: In this study, we determined the kinetics of PAF production in cell- vs. MVP over time. IL-8 release assay was further used to confirm the PAF-R-agonist activity in KBP cells using PAF as positive control. Moreover, we verified the PAF-R-agonist activity in UVB-MVP in animal models.

Results: The kinetics of PAF agonist production following UVB suggest that PAF-R agonists generated in response to UVB were cell-associated early, then, were found predominantly in MVP. The PAF-R-agonist activity found in MVP of HaCaT cells 2 h post UVB. UVB-MVP contain approximately 20 ng of PAF activity per 1E+10 MVP. However, PAF agonistic activity was not found in control MVP, and UVB-MVP did not generate IL-8 release in PAFR- negative KBM cells. Topical application of lipid extracts from UVB-MVP derived from HaCaT cells onto ears of WT mice

resulted in an increase in ear thickness at 2 h, however, there was no effect on PAF-R Knock-out (KO) mice

Summary/Conclusion: This study suggests that UVB-MVP contain bioactive PAF agonists involved in acute UVB-induced inflammation. This is the first study demonstrating that UVB-MVP carry PAF.

Funding: National Institutes of Health (NIH): R21 AR071110.

PF04.05

A mathematical model for extracellular vesicles, as a communication tool between cells.

Anna Concetta Berardi[a] and Andrea Collevecchio[b]

[a]ospedale Santo Spirito Pescara, Pescara, Italy; [b]Monash University, Melbourne, Australia

Introduction: The main goal of the present work is to introduce a mathematical model for extracellular vesicles (EV), as a communication tool between cells.

Methods: Our basic model has a graph theoretical representation in terms of weighted graphs and stochastic processes that take values on the vertices of the graph, which play the role of cells. More specifically consider a complete graph, where each vertex communicates with any other vertex. To each edge of the graph associate a positive number, which might be interpreted as the euclidean distance between cells. In order to understand the main features of the model, it is enough to isolate one designated cell, called the root, and understand how effective is its communication with the other cells.

Results: We regard the EV as signals sent to other cells. At each stage the root sends a signal to another cell chosen with probability proportional to the weight associated to the connecting it to the root. Each time an edge is traversed, its weight is updated. This allows learning during the communication. In other words, the root has preference in communicating with cells that has been already contacted before. Each signal contains a task. Once a cell receives a task, it will activate in order to complete it. On the other hand, the completion of the task has a random duration. If during this time the cell is contacted too frequently by the root cell (that is above a certain threshold), it will abort the task.

Summary/Conclusion: Our goal is to understand what are the phases transitions of this model with respect to its parameters as the number of vertices grow to infinity. In other words, if the threshold associated to the abortion is large enough, we expect to have a positive proportion of the cells to accomplish the task.

PF05: EVs in Infectious Diseases and Vaccines
Chairs: Tsuneya Ikezu; Maja Mustapic
Location: Level 3, Hall A

15:30–16:30

PF05.01

Extracellular vesicles from KSHV-infected cells stimulate antiviral immune response through mitochondrial DNA

Hyungtaek Jeon, Jisu Lee, Suhyuk Lee, Su-Kyung Kang, Sang June Park, Seung-Min Yoo and Myung-Shin Lee

Eulji University School of Medicine, Daejeon, Republic of Korea

Introduction: Interferon-stimulated genes (ISGs) are vital in controlling viral infections. As many antiviral ISGs continue to be identified, their roles in viral pathogenesis are also being explored in more detail. Kaposi's Sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma, which is the most common cancer in acquired immune deficiency syndrome patients. Because KSHV contains numerous viral proteins that modulate antiviral response, type 1 Interferon response is strongly suppressed in KSHV-infected cells. However, the antiviral effects of extracellular vesicles (EVs) during *de novo* KSHV infection have not been investigated to our best knowledge.
Methods: EVs were isolated from KSHV-infected cells at 24 h of postinfection and characterized. The expression of ISGs in these EVs-treated human endothelial cells was investigated and underlying mechanisms were analysed.
Results: In this study, we showed that KSHV-infected cells induce ISG response in uninfected bystander cells using EVs. mRNA microarray analysis indicated that ISGs and IRF-activating genes were prominently activated in EVs from KSHV-infected cells (KSHV EV)-treated human endothelial cells, which were validated by RT-qPCR. Mechanistically, mitochondrial DNA on the surface of KSHV EVs was presumed to be associated with ISG response via the cGAS-STING pathway. In addition, KSHV EV-treated cells showed lower infectivity for KSHV and viral replication activity than mock EV-treated cells.
Summary/Conclusion: Our results indicated that EVs from KSHV-infected cells would be an initiating factor for the innate immune response against viral infection, which would be helpful to expand our understanding of the microenvironment of virus-infected cells.
Funding: This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF-2017R1A2B1006373, NRF-2017R1A2B4002405).

PF05.02

Exosomes secreted by platelets infected with Hepatitis E virus can mediate transmission of HEV

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Introduction: Evidences suggested that exosomes can transfer genetic material between cells, including viral nucleic acids, proteins or miRNAs which can mediate transmission of viruses such as HBV or HCV. It is known that platelet-derived exosomes constitute the major fraction in the circulating plasma which can participate in haemostasis, immunity and development. Whether the virus infected platelet-derived exosomes can also promote the transmission of virus has not been reported. The hepatitis E virus (HEV) is one of the most common causes of acute hepatitis worldwide. Recent studies have shown that the exosomes secreted by HEV-infected cells were infectious. Our studies have confirmed that HEV can infect platelets, thus we conducted this study to prove if exosomes secreted by platelets infected with HEV are also infectious, thereby further promoting the transmission of HEV.
Methods: An *in vitro* model of HEV-infected platelets were established by HEV-G3 virus strain and washed human platelets and the exosomes were isolated from HEV-infected and uninfected platelet by differential centrifugation and magnetic bead separation. Exosomes were characterized by Western Blot and TEM, and quantitated by NTA. qRT-PCR and ELISA were used to detect HEV RNA and proteins in exosomes. Positive exosomes were used to infect PLC/PRF/5 cells, observing the changes of HEV RNA and proteins within one month.
Results: The *in vitro* model of HEV-infected platelets was successfully established. The concentration of exosomes secreted by HEV-infected platelets was higher than uninfected platelets. Exosomes isolated from HEV-infected platelets contained HEV RNA and

proteins. HEV RNA and proteins were detected in cells and supernatant of PLC/PRF/5 cells infected with positive exosomes, and the concentration of which increased after the culture of one month.
Summary/Conclusion: Our study showed that HEV can promote the secretion of platelet exosomes and these vesicles can establish a productive infection which suggested that the exosomes secreted by platelets not only play a role in haemostasis, immunity and development, but also play a non-negligible role in the transmission of the virus.
Funding: 1.CAMS Innovation Fund for Medical Sciences (CIFMS2016-I2M-1-018)
2. Supported by the Fundamental Research Funds for the Central Universities(Item No:3332018125)

PF05.03

Multi organ association mediated by extracellular vesicles secreted from HBV positive hepatocyte

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Introduction: Hepatitis B virus (HBV) infected hepatocytes secreted extracellular vesicles such as virion, exosome and incomplete virions such as hallow particles which have only HBs viral antigens but neither capsid and HBV genome. We found that the EVs are taken by monocyte/macrophage which upregulates PD-L1, immune checkpoint molecule. (Kakizaki et al PLOS one in press). It suggests that the EVs play critical roles in viral immunology.
Methods: PKH labelled EVs are injected into mice then those biodistribution are analysed by tissue sections and confocal microscopy and flow cytometry then multiorgan association are analysed by use of bone marrow transplantation. The HBV hepatitis model mice was established to analyse the significance of the EVs on HBV hepatitis.
Results: In almost all the organs we analysed the PKH signal were detected which suggest the biodistribution of the EVs are systemic. Especially in the bone marrow monocytes, PKH positive cells are abundant. Then the bone marrow cells which were treated by PKH labelled the EVs secreted from HBV infected hepatocytes were transplanted. The organ where the most PKH positive cells were detected was gut. These cells showed CD103 positive which suggest these cells are regulatory DCs. The HBV hepatitis model mice treated by the EVs showed persistent HBV infection.
Summary/Conclusion: Based on the results, the EVs secreted from HBV infected hepatocytes have significant roles in HBV hepatitis through the multiorgan association of liver, bone marrow and gut.
Funding: AMED hepatitis grant.

PF05.04

HIV-1 Nef mediated Hck kinase activation triggers loading of TACE into EVs in a ceramide-dependent manner

Zhe Zhao, Riku Fagerlund and Kalle Saksela

University of Helsinki, Helsinki, Finland

Introduction: TNF-α converting enzyme (TACE) exists in circulating EVs collected from HIV-infected individuals. In addition to its role in TNF-α shedding, TACE is responsible for the proteolytic maturation of numerous cytokines, cytokine receptors and extracellular matrix components involved in inflammation. Thus, EV-associated TACE could be a key player in the chronic immune activation that drives AIDS pathogenesis and persistent viral replication. Although uploading of TACE into EVs is promoted by the HIV-1 pathogenicity factor Nef, the mechanism of TACE secretion remains incompletely understood.
Methods: EVs were isolated by ultracentrifugation and analysed by western blotting. Gene knock-out by CRISPR was used to study TACE loading into exosomes.
Results: We have previously shown that uploading of TACE into EVs is promoted by HIV-1 pathogenicity factor Nef via its interaction with cellular protein kinases. Herein, we have studied the molecular mechanisms of TACE loading into EVs in more depth and show that active Src family tyrosine kinases (SFKs) Lck, Hck, Lyn, Fyn and Fgr can trigger TACE secretion. Among the SFKs Hck is unique by displaying two isoforms p59 and p61. We found that only activated p59, but not p61, could trigger TACE secretion. In contrast to the myristoylated and palmitoylated SFKs and p59Hck the p61 isoform lacks the palmitoylation signal and these differing signals have been shown to direct p59 and p61 to the plasma membrane and lysosomes, respectively. Our observations show that catalytic activity and proper membrane association domain of Hck is required for TACE secretion into EVs. We characterized the origin of the EVs in Hck-activated TACE secretion by nSmase2 knock-out, and found that Hck-mediates the loading of TACE into vesicles that similar to classical exosomes are generated in a manner requiring nSmase2-mediated ceramide synthesis.
Summary/Conclusion: We conclude that HIV-1 Nef mediated Hck p59 kinase activation triggers the

loading of TACE into exosome-like vesicles in a ceramide dependent manner.
Funding: Jane and Aatos Erkko Foundation

PF05.05

Role of Extracellular Vesicles in HIV and Methamphetamine induced neurotoxicity

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Introduction: The advent of combined antiretroviral treatments (cART) has markedly decreased the prevalence of HIV-associated dementia. However, there remains a high prevalence rate of the milder forms of HIV-associated neurocognitive disorders (HAND). Although many contributing factors have been studied, the role of drugs of abuse has remained elusive. Methamphetamine (Meth) and related amphetamine compounds, which are potent psychostimulants, are among the most commonly used illicit drugs. Long-term Meth abuse is associated with a host of systemic and neurological maladies. Neurologically, Meth abusers exhibit cognitive and psychomotor impairment, and have shown increased risk for HIV infection. However, the mechanisms underlying Meth and HIV neurotoxicity are still not known. This study focuses extracellular vesicles (EVs) and their role in HIV infection and chronic Meth abuse. Our results presented here, indicate that Meth can not only increase EV biogenesis and release but also change the composition of EV cargo.
Methods: EV isolations, EV quantification by Nanoparticle tracking analysis, Immunoflurescence and structural illumination microscopy, transmission electron microscopy, Taqman RT-PCR, *In situ* hybridization, *in vitro* primary macrophage cultures.
Results: Nanoparticle tracking analysis and transmission electron microscopy revealed that Meth changed EV dynamics in uninfected and HIV infected macrophage cultures. Our investigation revealed that the genes involved in the endosomal sorting complexes required for transport (ESCRT) are responsible are significantly increased upon Meth treatment. Further, our data reveals that Meth increases the release of HIV accessory protein, myristoylated Nef (Myr-Nef), that plays a critical role in HIV/AIDS progression. Myr-Nef is N-terminally myristoylated, which acts as a membrane anchor. Furthermore, we also reveal that gp120 is released in the EVs along with Myr-Nef. We show that the release of gp120 is followed by the increase in syncytia formation in the macrophage cultures.
Summary/Conclusion: We conclude that chronic Meth abuse interferes with EV biogenesis and cargo release in HIV infected cells. These results can uncover the role of chronic Meth abuse in progression of HIV pathogenesis.
Funding: NIH/NIMH/NIDA

PF05.06

Extracellular vesicle-associated cytokines in HIV infected human lymphoid tissue *ex vivo*

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Introduction: Cytokines play an important role in HIV infection. Some of these cytokines are present on the surface or encapsulated in extracellular vesicles (EVs). We investigated the modulation of EV-associated cytokines during HIV infection and antiretroviral therapy (ART) in human *ex vivo* tonsils.
Methods: *Ex vivo* tonsils were infected with HIV-1 strains, X4-LAI04 or R5-SF162. HIV was either allowed to replicate for 15 days, or tissues were treated with ART (3TC and AZT) at day 2 post-infection. 33 cytokines in soluble or EV-associated forms were evaluated with multiplexed bead-based assays.
Results: Infection with HIV-1 led to increases in many soluble cytokines, with the highest increases in IL-2, IL-12, IL-15, IFN-γ, MIP-1α, MIP-1β and RANTES by both viruses. These same, and additional, cytokines, were elevated in EV-associated form, often increasing in both EV surface and internal compartments. Likewise, soluble cytokines unaffected by HIV infection tended to also be unchanged in EVs.

ART treatment halted HIV-1 replication, but cytokines increased by HIV infection remained elevated. After 13 days of ART, five of the above seven soluble cytokines remained high for X4, and 4 for R5. EV-associated cytokines were less likely to be restored: 13 days after ART, for X4 all 10 of the most upregulated cytokines remained high, and for R5 7 of 10.
Summary/Conclusion: Cytokine levels increased during HIV infection in both soluble and EV-associated forms; the same cytokine is often upregulated in both forms. Many soluble cytokines upregulated by HIV did

not decrease even after 13 days of ART, and EV-associated cytokines were even less likely to decrease. X4 induced increases were less likely to return to control levels. ART-treated infected human tissues provide a new model to study tissue activation after HIV replication is suppressed, in particular, the role of EVs in this phenomenon. These studies will assist in deciphering mechanisms of pathologies that develop in ART-treated patients.

PF05.07

Circulating MiR-122 and let-7a may predict progression to hepatocellular carcinoma in patients with chronic hepatitis C virus infection

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Introduction: At the bedside, circulating microRNAs in human body liquids are noted as `liquid biopsy' to evaluate the disease stages, especially in Liver diseases, `actual biopsy' is used in patients because of its invasiveness. In chronic liver disease, many circulating miRNAs are recently reported such as miR-122, miR-192, miR-223 to diagnose HCC patients and HCV- or HBV-infected patients, in this study. To identify the miRNAs which can predict HCC (hepatocellular cell carcinoma) development, we first focused on the microRNAs associated with the liver fibrosis, because liver fibrosis stages are one of the most important factors to predict Hepatocellular cell carcinoma (HCC) development and evaluated those predictable ability among other noninvasive fibrosis markers to clarify our hypothesis that those miRNAs are pathologically related to carcinogenesis development.
Methods: Serum circulating miR-122 and let-7a were retrospectively evaluated using RT-PCR in 35 patients with HCV-related chronic hepatitis and cirrhosis who undergone DAA treatment. HCC had developed in 8 patients afterwards in the observation period. Informed consent was obtained and the study was approved by a recognized medical ethics committee in our hospital.
Results: Serum miRNA miR122and let-7a levels were significantly higher in liver chirrosis than chronic hepatitispatients. (miR122, p = 0.00836 let-7a p = 0.01595). For the predictable ability of HCC, AUROC of miR122 was 0.85606 and le1-7a was 0.76667,which showed highest ability compared with other non-invasive fibrosis markers, such as APRI, FIB-4. (AUROC = 0.5023, 0.66697, respectively) Based on our ROC results to predict complicating HCC, COF of miR122 was 0.04175 (sensitivity and specificity of 86% and 75%, respectively) and COI of let-7a was 0.0166 (sensitivity and specificity of 71% and 87%). The cumulative incidence rate of HCC was significantly different miR-122 ≥ 0.04175 and > 0,04175 groups or let-7a ≥ 0.01666 (p = 0.050 and 0.00054)
Summary/Conclusion: Serum miR-122 and let-7a values appear to have superior ability to predict HCC development in patients with chronic HCV infection, which implies the possibility that they have crucial role in HCC development.

PF05.08=OWP2.07

Biogenesis of JC polyomavirus associated extracellular vesicles depends on neutral sphingomyelinase 2

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Introduction: JC polyomavirus is a non-enveloped virus that causes progressive multifocal leukoencephalopathy (PML) in immunocompromised patients. JCPyV infects cells by first binding to the major attachment receptor lactoseries tetrasaccharide C (LSTc), followed by the serotonin receptor 5-hydroxytryptamine type 2 required for entry. In PML, JCPyV undergoes lytic infection in oligodendrocytes and astrocytes, both of which have been shown to lack LSTc. Further, deep sequencing has shown that viral quasispecies existing in PML patients contain mutations in the sialic acid binding pocket of the major viral capsid protein, rendering these virions incapable of binding LSTc. We have recently demonstrated that JCPyV is packaged into extracellular vesicles (EV) that can spread the virus, potentially overcoming this paradox. Here, we begin to characterize the biogenesis of this EV-virus association by examining endosomal sorting complexes required for transport (ESCRT) proteins and neutral sphingomyelinase 2 (nSMase2).
Methods: Cambinol was used to specifically target nSMase2 activity. Knockdown cell lines were created with shRNA targeted against ALIX, TSG101, or SMPD3. SMPD3 was also targeted using CRISPR/Cas9 genetic knockout in separate cell lines. Knockdown was confirmed by qPCR and/or Western blot, and knockout by next generation sequencing. EV were concentrated by differential centrifugation and evaluated by transmission electron microscopy, Western blot, nanoparticle tracking analysis, infection, and qPCR for protected viral genomes. Infection was

scored by immunofluorescence analysis with antibodies against the major viral capsid protein VP1.

Results: We found that depletion of nSMase2 by cambinol, genetic knockdown or knockout caused a reduction in spread of JCPyV over time. Knockdown and knockout SMPD3 cell lines produced less infectious EV. In the absence of nSMase2, cells produced more EV but there were fewer protected genomes associated with the EV. Knockdown of Alix or TSG101 had no effect on the infectivity of EV or the production of EV.

Summary/conclusion: Overall, our studies found that biogenesis of JCPyV associated extracellular vesicles depends upon the enzymatic activity of nSMase2 and not the ESCRT-related proteins Alix or TSG101.

Funding: NIH R01NS043097

PF05.09=OWP2.08

Exosomes mediate the anti-viral activity of interferon-β against zika virus infection

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Introduction: IFNβ-induced exosomes (Exo-IFNβ) may impact on viral dissemination or antiviral immunity and therefore involve in the pathogenesis of many infectious pathogens. However, little is known about its underlying mechanisms. To better understand how Exo-IFNβ performs its anti-viral effect, we employed RNA sequencing analysis to explore the exosomal expression profiles of lncRNA and mRNA related to viral infections. We hypothesized that exosomes can regulate viral infection through transmitting enclosed specific lncRNAs into neighbouring cells to inhibit viral replication.

Methods: Exosomes were purified from A549 with/without IFNβ treatment by serial centrifugation followed by sucrose density gradient purification, and characterized by TEM and Western Blot. ELISA assay were performed on purified exosome fractions to demonstrate that they are free of IFNβ. ZIKV replication was assayed by real-time PCR.

Results: ZIKV replication was significantly suppressed in A549 cells pre-treated with Exo-IFNβ followed by ZIKV infection. Moreover, we found that anti-ZIKV effect of Exo-IFNβ is IFN-independent because ZIKV replication was also decreased in U5A cells (IFN-α/β receptor IFNAR deficient) pre-treated with Exo-IFNβ . Similar results were observed in Dengue virus and HCV infections. RNA sequencing analysis found several lncRNAs and mRNAs were differentially expressed and function annotation and pathway analysis demonstrated that the differentially expressed genes were involved in many functions and pathways, including anti-viral infection. To validate the RNA sequencing analysis results, some lncRNAs were selected to test their expression levels by qPCR. We are in the process of deciphering the mechanism employed by these exosomal lncRNAs in anti-viral activty independent of inteferon.

Summary/conclusion: We believe that understanding the anti-viral functional molecules wrapped in exosomes may help design exosomes as efficient vehicles for antiviral therapy.

Funding: Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (2016-12M-3–025)

PF06: Advances in EV Quantification and Characterization
Chairs: Estefanía Lozano-Andrés; Kenneth Witwer
Location: Level 3, Hall A

15:30–16:30

PF06.01

Exosome quantification by ELISA and Flowcytometry using anti-CD9 antibody

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Introduction: Quantifying and characterizing exosomes in a reproducible and reliable manner has been challenging due to their small sizes, of which the ranges are from 30 to 150 nm in diameter. The analysis used to be mainly performed with either the electric microscopy or the nanoparticle tracking analysis; however, these techniques are low throughput and not enough for the quantification especially in the large and heterogeneous populations. Also, attempts to analyse exosomes using traditional PMT-based flow cytometers has been hampered by the limit of detection of such small particles and low refractive index. Here, to overcome these limitations, we used the highly qualified and validated monoclonal antibodies for CD9 on the surface of exosome to employ ELISA and the high sensitive flow cytometry. In this study, we would like to show and discuss more reliable and robust platforms for the quantification of exosomes with use of ELISA and flow cytometry.

Methods: Malignant cell line-derived exosome was prepared by the ultracentrifugation

↓

Diluted the samples in PBS at 1:60, 1:120, 1:240, 1:480 and 1:960

↓

Measured the samples either by CD9-based ELISA (Hakarel Inc) or Flow cytometer (CellStream, Luminex Corporation)

Results: The quantifications of exosomes were performed by ELISA and CellStream flow cytometer with use of anti-CD9 monoclonal antibody

Summary/Conclusion: In this study, the quantifications of exosomes were performed by ELISA and CellStream flow cytometer with use of anti-CD9 antibody. Tumour cell-derived exosomes were labelled with CD9-PE. The average concentration of the exosomes was measured by CD9 ELISA whereas the mean fluorescence intensity and the objects per microlitre for the exosomes and control samples were shown by CellStream flow cytometer. The robust sensitivity of ELISA and CellStream flow cytometer with use of the validated CD9 antibody would provide an informative platform for measuring exosomes.

Funding: No fundings.

PF06.02

Characterizing the light-scatter sensitivity of the CytoFLEX flow Cytometer

George Brittain, Sergei Gulnik and Yong Chen

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Introduction: Extracellular vesicles (EVs) and other biological nanoparticles (NPs) generally fall within the optical noise of light-scatter-based detection methods, and most flow cytometers are not sensitive enough to effectively detect NPs less than 300 nm in diameter. The CytoFLEX is a notable exception to this: it is so sensitive that the SSC detector actually has an attenuation filter to reduce 95% of the scatter signal, adjusting it to a range useful for cells. As an alternative, the Violet SSC (VSSC) signal is unfiltered and can be used to bring the CytoFLEX sensitivity well into the nanoparticle range. However, the added VSSC layer can confuse individuals, and a few instrument comparisons have even been published by users unfamiliar with the use of VSSC on the CytoFLEX.

Methods: In order to better characterize the biological threshold sensitivity of the CytoFLEX using VSSC, we analysed a variety of NPs of different compositions, including viruses and purified plasma EVs. The plasma EVs were prepared from fresh human blood using centrifugation, size filtration, and column chromatography, followed by size characterization using DLS. After acquisition on the CytoFLEX, we converted the median scatter intensity for each sample to either their size or refractive index (RI) using Mie theory approximations.

Results: We found that the CytoFLEX could fully resolve 70 nm polystyrene and 100 nm silica (Si) NPs, including Si with a RI of 1.43 at 405 nm. We could fully resolve both 110 nm MLV viruses and 90 nm Adenoviruses with RIs of 1.47–1.50. And, we were

able to detect plasma EVs at least as small as 80 nm in diameter using only a VSSC trigger, though immunofluorescence was necessary to fully resolve the smallest of these EVs from noise.

Summary/Conclusion: Ultimately, the CytoFLEX is highly sensitive for NP detection. Moreover, unlike dedicated microparticle analysers, the CytoFLEX is a full-fledged flow cytometer with a biological dynamic range extending from approximately 80 nm–50 μm.

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PF06.03

Preparing a CytoFLEX for Nanoscale flow Cytometry

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Introduction: Built around semiconductor technology, with a number of innovations to enhance light capture, reduce noise and prevent signal losses, the CytoFLEX is capable of detecting biological nanoparticles (NPs) as small as 80 nm by light scatter, and has a linear fluorescence range that extends down into the single digits for fluorophores like FITC. However, in order to properly setup the CytoFLEX for NP analyses, a variety of considerations need to be taken into account, some of which are extraordinary to conventional flow cytometry.

Methods: In this poster, we will demonstrate how to properly setup and clean a CytoFLEX flow cytometer for NP analyses. First, we will explore the different threshold options and sensitivity ranges. Next, we will show how to clean the instrument and reduce noise. And finally, we will discuss several important issues that affect proper sample analyses.

Results: The three primary detection methods on the CytoFLEX are FSC, SSC and Violet-SSC (VSSC). FSC on the CytoFLEX utilizes comparative signal analyses rather than traditional small-angle scatter, and is accurate for sizing events from 500 nm to 50 μm, independent of the refractive index or membrane integrity. The biological threshold sensitivities for SSC and VSSC on the CytoFLEX range roughly between 250 nm–20 μm and 80 nm–2 μm, respectively. In order to take full advantage of the lower end of these scatter ranges, cleaning the instrument and thoughtful sample preparation are very important.

Summary/Conclusion: Ultimately, the CytoFLEX is one of the most sensitive flow cytometers on the market. However, with such great power comes great responsibility to properly prepare the instrument and samples for effective nanoscale flow cytometry experiments.

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PF06.04

Improved scatter sensitivity of a flow cytometer for detection of extracellular vesicles

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Introduction: To investigate the biomarker potential of extracellular vesicles (EVs), EV subtypes are studied by flow cytometry. A flow cytometer detects fluorescence, forward (FSC) and side scattered (SSC) light of single EVs. However, the scatter intensities of the majority of EVs are below the detection limit of common flow cytometers because EVs are small and have a low refractive index. We aim to improve the scatter sensitivity of a common flow cytometer 450-fold for SSC and 10^7-fold for FSC, which will allow detection of 100 nm EVs. Improved scatter sensitivity enables us to derive the size of EVs from the scatter signal and to increase the fraction of EVs that can be characterized using immunofluorescence as well as scatter-based sizing.

Methods: A FACSCanto (Becton Dickinson) was adapted by replacing the 20 mW laser with a 20–200 mW adjustable power laser (both 488 nm Sapphire, Coherent). Confocal detection was achieved by replacing the standard 1000 μm pinhole on SSC by a 200 μm pinhole, and the standard photodiode on FSC by a 350 μm pinhole and PMT. The improvements in scatter sensitivity were quantified by calculating the scatter stain index (SI) (median intensity of a bead minus median intensity of the noise divided by two times the standard deviation of the noise) of a 500 nm polystyrene bead and the robust coefficient of variation (rCV) of a 100 nm polystyrene bead (both BioCytex). Ideally the SI is as high as possible and rCV as low as possible.

Results: A 10-fold increase in laser power increased the SI on SSC 2.9-fold and on FSC 20-fold, whereas the rCV improved (reduced 0.67-fold and 0.97-fold, respectively). The improved confocal detection increased the SI on SSC 6.4-fold and on FSC 550-fold, while the rCV slightly worsened (increased 1.1-fold and 1.02-fold, respectively). Combining both increased laser power and confocal detection resulted in a 20-fold increase in SI for SSC and 2 · 10^4-fold for FSC, and improved the rCV (reduced 0.39-fold and 0.24-fold, respectively).

Summary/Conclusion: Adaption of the optical configuration of the FACSCanto by increasing the laser power and confocal detection improved the scatter sensitivity 20-fold for SSC and 2 · 10^4-fold for FSC. Next, we will evaluate the influence of increased measurement time and reduction of the number of particles in the sheath on the scatter sensitivity.

Funding: NWO-TTW Perspectief CANCER-ID 14195

PF06.05

Lipoprotein particles can be detected by high-resolution flow cytometry and potentially interfere with EV characterisation

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Introduction: Lipoproteins co-isolate with EVs and are potential confounders in EV characterisation. CD36 is a membrane-bound scavenger receptor located on cells and EVs capable of interacting with VLDL and LDL, which could interfere with antibody-based phenotyping. Freezing and thawing samples was shown to increase phosphatidylserine-positive (PS+) EVs while other common phenotype markers were unchanged. This could provide a method for disrupting lipoproteins and EVs. Thus, we aimed to investigate the impact of lipoproteins on EV characterisation and freezing/thawing samples on their dissociation from EVs on a high-resolution flow cytometer (hFCM).

Methods: Plasma from 6 healthy individuals was subjected to either 0, 2, 4 or 6 freeze-thaw (FT) cycles and stained with a cocktail of lactadherin-FITC, anti-CD41-BV510, anti-CD36-PE and anti-ApoB-APC or lactadherin-FITC and matched isotype controls. Samples were analysed on an Apogee A60 Micro-PLUS hFCM. Gating was performed as follows: size gates established on silica reference beads; phenotype gates set on 99th percentile of isotype control channel fluorescence.

Results: hFCM was able to detect both free apolipoprotein B (ApoB) particles and ApoB bound to PS+CD41+, PS+CD36+ and PS+CD41+ CD36+ EV phenotypes. From 0–2 FT cycles, ApoB bound to PS+CD41+ and PS+CD41+ CD36+ phenotypes tended to decrease ($p > 0.05$). Moreover, ApoB bound to PS+CD36+ increased 4.9-fold from 0–2 FT cycles for ($p < 0.05$). Interestingly, this progression mirrored that of PS+CD36+ (2.0–2.5-fold, $p < 0.05$), bulk CD36+ (1.8–2.4-fold, $p < 0.05$) and ApoB+ (4.1–5.0-fold, $p < 0.01$). Finally, in line with previous reports, PS+ tended to increase following FT (1.5-2.1-fold, $p > 0.05$). Contrary to previous reports, certain EV phenotypes decreased from 0–2 FT cycles (PS+CD41+ and PS+CD41+ CD36+, both 2.6-fold, $p < 0.05$) suggesting that EV phenotypes might perish following FT further confirmed on bi-variable plots of data.

Summary/Conclusion: This study demonstrates that ApoB can be detected on hFCM and thereby interfere with EV characterisation. What further complicates matters is that lipoproteins could carry markers traditionally associated with EVs including PS and CD36. FT cycles did not consistently dissociate EVs and lipoproteins; however, FT affected certain EV populations. Further studies are required to elucidate these findings.

PF06.06

Analysis of fluorescent labelling efficiency of extracellular vesicles derived from different kingdoms of life with lipid-binding dyes via nano-flow cytometry

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Introduction: In all domains of life – archaea, bacteria and eukarya, cells produce and release extracellular vesicles (EVs). The double-layered lipid membrane is the most prominent feature of EVs, and fluorescent labelling with lipid-binding dyes has been frequently used to visualize and detect single EVs. For example, most conventional flow cytometers rely on fluorescence threshold triggering for single EV detection upon membrane labelling with lipophilic dyes. However, the labelling efficiency of EVs with these lipid-binding dyes remains unknown. Here, we reported an approach to quantitatively analyse the labelling efficiency of lipid-binding dyes toward EVs by using a laboratory-built nano-flow cytometer (nFCM) that enables light scattering detection of individual EVs as small as 40 nm.

Methods: EVs were extracted from cultured medium of HCT15 cells (colorectal cancer cell line), *E. coli* O157:

H7 (gram-negative), *S. aureus* (gram-positive) and *Prochlorococcus* (Pro., marine cyanobacteria) by differential ultracentrifugation. EVs isolated from E. coli O157:H7 and *S. aureus* were further purified by floatation in iodixanol density gradient. The purity of these EV isolates was assessed by enumerating the particles before and after the treatment with Triton X-100. Subsequently, the labelling efficiency of several lipophilic fluorescent dyes, such as PKH26, PKH67, DiI and Di-8-Ane for EVs were evaluated by comparing with their light scattering signals.

Results: The purity of EVs isolated from HCT15 cells, *E. coli* O157:H7, *S. aureus* and Pro. were around 80% to 90%. Compared with side scattering signals, we found that almost all the EVs derived from *E. coli* O157:H7, *S. aureus* and Pro. could be lightened up by PKH26, PKH67, DiI and Di-8-Anepps. However, only around 40% of EVs isolated from HCT15 cells could be labelled by these dyes. Morphological study by cryo-TEM indicates that some vesicles secreted by HCT15 cells had surface protrusions (electron-dense spikes protruding from the membrane). We suspect this structure may prevent these lipophilic dyes from intercalating with EV membrane.

Summary/Conclusion: The nFCM provides a straightforward platform to analyse the labelling efficiency of EVs with different lipid-binding dyes, which will be very helpful in guiding the development of efficient vesicle-labelling strategies.

PF06.07

Evaluating the surface charge of yeast extracellular vesicles as a function of environmental parameters

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Introduction: Understanding the mechanisms of extracellular vesicle (EV) fate and transport is critical to predicting their targeting capabilities and delivery efficiencies. Surface chemistry has been shown to be an effective predictor of the fate of nanomaterials (which include EVs) in complex environments. In particular, ascertaining how surface charge changes based on surrounding conditions provides a foundation for the prediction of nanomaterial behaviour. Hence, the goal of this study is to evaluate EV surface charge as a function of environmental parameters to predict their ultimate environmental fate.

Methods: EVs were isolated from yeast (*S. cerevisiae*) cell culture via the ultracentrifugation/density gradient purification method. Nanoparticle Tracking Analysis (NTA), transmission electron microscopy (TEM) and the Coomassie protein assay data collectively confirm the presence of EVs. To evaluate the surface charge of EVs, electrophoretic mobility was measured (Malvern Zetasizer Nano ZS) at varied pHs, ionic strengths and organic contents to simulate environmental solution chemistry; values were then converted to zeta potential estimates via the Smoluchowski approximation.

Results: Initial tests reveal EVs to have a predominantly negative charge, with a zeta potential of −5.4 mV in phosphate buffer. Higher ionic strengths destabilize vesicles, causing aggregation by neutralizing the surface charge.

Summary/Conclusion: We demonstrate an initial understanding of the behaviour of how EV surface charge is influenced by various environmental parameters; the effects of these changes are variable. This implies that studying these trends mechanistically in complex systems may be challenging. Changes to the EV surface chemistry induced by alterations in the surrounding environment often also causes aggregation, which has implications for fate and transport. Further, work will be performed to probe the aggregation tendencies of EVs. The quantification of physicochemical parameters is a first step in parameterizing future fate and transport models.

Funding: Funded by the National Science Foundation (NSF) and the Environmental Protection Agency (EPA) under NSF Cooperative Agreement EF-0830093 and DBI-1266252, Center for the Environmental Implications of NanoTechnology.

PF06.08

Isolation and characterization of bovine milk-derived EVs.

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Introduction: Extracellular vesicles (EVs) are secreted from various cells and known to contain DNA, RNA and protein. Such inclusion is taken in other cells and plays functionally. Since recent studies reported that EVs are detected in food, such as fruits, vegetables and bovine milk, we hypothesized that functional EVs in food could contribute to human health. In the study, we investigated whether the growth environment for dairy cattle affected the contents and functions of EVs from bovine milk.

Methods: Milk was warmed at 37°C water bath for 10 min, then mixed with 1/100 volume of acetic acid at room temperature for 5 min and centrifuged at

10,000 x g at 4°C for 10 min to remove milk fat and debris. The supernatant was filtered with a 0.22 um membrane and defined as whey. The whey was ultracentrifuged at 200,000 x g for 70 min at 4°C. After PBS wash was performed twice, the pellet of EVs was resuspended in PBS, and centrifuged at 10,000 x g for 5 min at 4°C. The supernatant was used as EV solution. Particle size and concentration of EVs were measured by qNano. Total RNA of EVs was isolated by miRNeasy Mimi kitand the RNA concentration was measured by Agilent 2100 Bioanalyzer. RNA sequence was performed by Ion S5. The sequences data was analysed by CLC Genomics.

Results: We compared two bovine milks, which were collected from different farm. Milk A and milk B were both from healthy cattle who grew up with nutrient-filled pasture without giving stress, however, B was raised under better conditions. Between milk A and B, bovine milk-derived EVs were almost same particle size and concentration. Then, amount of RNA containing EVs were same between milk A and B. However, NGS data was revealed that EVs from milk B contained more immune-related microRNAs than milk A.

Summary/Conclusion: This study revealed that the better growth environment of dairy cattle increased immune-related microRNAs in bovine milk-derived EVs and so might be better for health.

PF06.09

Regulatory effect of apple-derived nanoparticle on intestinal organic anion transporting polypeptide (OATP) 2B1

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Introduction: Several studies have shown that plant-derived nanoparticles (NPs) taken up by the intestinal cells affect intestinal function. Food-derived NP is known to facilitate delivery of proteins, nucleic acids including microRNA (miRNA) and other large molecules to intestinal tissues. Therefore, such large molecules may affect gastrointestinal functions through NPs. Accordingly, we investigated the effect of apple-derived NP to intestinal transporters through containing cargos.

Methods: NP was prepared by ultracentrifugation. Lipid membrane of NP and apple-derived nucleic acid were labelled by fluorescents to examine uptake in Caco-2 cells using microscope. Expressions of mRNA and protein of transporters in Caco-2 cells were evaluated by qRT-PCR and Western blotting. Transport activity of OATP2B1 was evaluated by uptake of oestrone sulphate. Apple miRNA targeting OATP2B1 predicted by *in silico* analysis were detected by RT-PCR. microRNA target sites for OATP2B1 were evaluated by deletion assay and luciferase assay.

Results: Fluorescent labelled NP and nucleic acids were observed in Caco-2 cells after 6 h exposure. NP significantly decreased expression and transport activity of OATP2B1 in Caco-2 cells. When NP were heat-denatured or broken by sonication, their decreasing effects were attenuated. In deletion assay, decrease of OATP2B1 mRNA expression was observed in only plasmid construct containing 3' untranslated region (3'UTR). Luciferase activity of pGL-OATP2B1-3'UTR was reduced by NP exposure. Seven miRNAs which predicted to bind to this region were detected in NP. Moreover, decreased luciferase activity was inhibited by some miRNA inhibitors for predicted miRNAs.

Summary/Conclusion: Apple NP reduced mRNA and protein expressions and activity of OATP2B1, suggesting that apple miRNA in NP is involved in drug food interaction. Moreover, it was suggested that apple miRNA contributes to drug disposition by regulation of drug absorption mediated by OATP2B1 through NPs,

PF06.10

Fluorescent retroviruses as reference particles for Nanoscale flow cytometry

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Introduction: Nanoscale flow cytometry (NFC) is a promising tool for phenotypic analysis of individual small particles such as extracellular vesicles (EVs) and viruses that are smaller than 500 nm in diameter. However, since many small EVs are currently at the limit of detection for commercial flow cytometers, successful detection of EVs requires optimization of both sample preparation and instrument settings. These optimizations require reference particles reflecting size, refractive index (RI), and fluorescence emission intensity of the labelled EVs of interest. Murine leukaemia virus (MLV) is a retrovirus ~114 nm in diameter as measured by cryo-EM, with an estimated RI of 1.5. Here we showcase the monodispersed nature of these viruses and demonstrate their use as fluorescence reference particles for NFC.

Methods: We engineered MLVs to express its envelope glycoprotein fused to green fluorescent proteins (eGFP and sfGFP) on the viral surface. MLVs were characterized by NFC and by nanoparticle tracking analysis. Because MLVs are monodispersed, we combined scatter intensities and hydrodynamic diameter to obtain the effective RI by solving the inverse light scattering problem using Mie theory.

Results: We measured an antigen density of ~300 MESF of GFP per virion. In addition, we found that antibody labelling of this virus-associated antigen with different fluorophore conjugates (PE, BV421 and AF647) modulates both scatter intensities and hydrodynamic diameter of the labelled virus. With regard to the hydrodynamic diameter, we show that the effective RI of the viruses could be tuned by using different fluorophores.

Summary/Conclusion: MLVs are similar to small EVs in size with equivalent surface area and comparable capacity for antigen expression. Unlike synthetic beads, MLVs can be genetically engineered to express protein antigens of choice in biologically relevant and consistent levels to act as internal positive controls for phenotypic studies of EV surface marker expression. Moreover, MLVs are monodisperse and have tuneable RI. Collectively, these properties support that MLVs are strong candidates as fluorescence reference particles for NFC.

Funding: Natural Sciences and Engineering Research Council of Canada (NSERC)

PF07: Biogenesis II
Chairs: Mathilde Mathieu; Hang Hubert Yin
Location: Level 3, Hall A

15:30–16:30

PF07.01

Proteomic profiling of outer membrane vesicles derived from MicA, a small RNA from *Escherichia coli*

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Introduction: Outer membrane vesicles (OMVs) produced by Gram-negative bacteria are utilized as vaccine or drug delivery platforms in terms of their efficient immune responses to host cells. In a previous report, we identified that ectopic expression of MicA, a small noncoding RNA from *E. coli*, produced a high production of OMVs as a conserved manner in both *E. coli* and *Salmonella* through both up- or down-regulation of OmpC or OmpA level, respectively, in OMV fractions. Moreover, MicA-derived OMVs showed the protective role against *Salmonella* challenge, suggesting that OmpC-enrichment in OMVs is important for the production and function of OMVs. However, MicA overexpression in the knockout strain of *ompA*, a target of MicA, still strongly induced the production of OMVs, indicating that another underlying mechanism of high production of OMV is presented.
Methods: Analysis of total and surface proteins from control- and MicA-derived OMVs from *E. coli* was performed using high-resolution mass spectrometry. The OMVs were isolated from culture supernatants, followed by characterization using Nanosight. We then analysed proteins of OMVs by in-gel digestion from SDS-PAGE, followed by nano LC-MS/MS analysis. The functional analysis of candidate proteins on the biogenesis of OMVs was performed by OMV preparation, BCA quantification, and protein analysis from knockout strains of specific genes.
Results: We found that spherical OMVs were an average diameter of 84.7 ± 1.3 nm and 88.2 ± 2.4 nm for MicA- or control-derived OMVs, respectively. Further, we identified 1,102 (38) or 656 (40) proteins for MicA- or control-derived OMVs in total (or surface) fractions are presented. Among them the level of 84 or 15 proteins from total or surface fractions, respectively, was decreased or absent compared to control sample. Total 99 proteins were categorized into 19 functional groups and found that 60 proteins are associated with flagella, ribosome, and modification. Moreover, the role of individual proteins on the biogenesis of OMVs using knockout strains expressing proteins was evaluated.
Summary/Conclusion: All our results enabled us to elucidate the underlying mechanism of high production of OMVs by MicA and the information will be utilized as a vaccine platform for infectious diseases.

PF07.02

Dysfunction in an autophagy-lysosome degradation pathway promotes secretion of ubiquitinated proteins via extracellular vesicles

Toshihide Takeuchi, Satoko Sakai, Harue Ando and Yoshitaka Nagai

Osaka University, Suita, Japan

Introduction: Autophagy-lysosome degradation is a cellular protective mechanism that prevents aberrant accumulation of cellular proteins, and thus, maintains protein homeostasis. Recent studies have suggested that autophagy impairment leads to an increase in secretion of aggregation-prone proteins, such as proteins that are associated with the neurodegenerative diseases, although molecular mechanisms underlying such secretion and its biological significance still remain elucidated.
Methods: The extracellular vesicle (EV) fractions were collected from the cell culture media by ultracentrifugation, and analysed by Western blotting, electron microscopy and nanoparticle tracking analysis.
Results: Here we show that perturbation of the autophagy/lysosome pathway activates secretion of ubiquitinated proteins via EVs. We found that treatment of cells with autophagy inhibitors leads to an increase in the amounts of ubiquitinated proteins, as well as autophagy-related proteins including LC3 and p62, in the EV fraction of the culture media. We also found that inhibitor treatment facilitates secretion of EVs distinct from exosomes in size, and that these EVs are involved in secretion of ubiquitinated proteins. Interestingly, analysis of knockout cells deficient for autophagy-related proteins revealed that the factors in the initiation step of autophagy are needed for EV-mediated secretion of ubiquitinated proteins.

Summary/Conclusion: These results indicate that autophagy impairment promotes secretion of ubiquitinated proteins via EVs. Our data provide the mechanistic link between the autophagy/lysosome pathway and vesicle secretion. We propose that cells may use the EV-mediated secretion as an alternative pathway to maintain protein homeostasis when cellular proteostasis machinery is functionally impaired.

Funding: This work was supported by JST; by KAKENHI (18H02585); by The Asahi Grass Foundation and The Tokyo Biochemical Research Foundation.

PF07.03

Identifying the miRNAs associated with EV Secretion from cancer cell lines

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Introduction: Extracellular vesicles (EVs) derived from cancer cells contribute to their surrounding microenvironmental cells for their benefit. Our group has previously shown that inhibiting the EVs production attenuated the angiogenesis in the tumour, resulting in the suppression of metastasis. Thus, understanding the mechanisms of EV secretion might contribute to the regulation of EV-mediated cancer progression. However, the precise mechanism of EV secretion in cancer cells remains unclear. The purpose of this study is to elucidate the unknown mechanisms of EV secretion in cancer cells. To reveal this, microRNAs (miRNAs), which regulate multiple genes, are employed.

Methods: To identify the EV secretion associated miRNAs, miRNA-based screening method was established. Combined with ExoScreen, which is ultra-sensitive detection method of EV by measuring surface protein of EVs, such as CD9 and CD63, miRNA-based screening was performed in colorectal cancer cell line, HCT116, and lung cancer cell line, A549. The results of the screening were confirmed by the nanoparticle tracking analysis. Candidate genes of these miRNAs were chosen by *in silico* analysis.

Results: From the initial 1728 miRNAs, we identified 13 miRNAs which are associated with EV secretion in each cell lines. Then, the target genes of these miRNAs were identified and confirmed that EV secretion was attenuated by siRNAs against candidate genes. From 6 miRNAs, 27 genes, which were associated with EV secretion, were identified. Interestingly, among six miRNAs, four miRNAs altered the EV secretion in both cell lines, HCT116 and A549.

Summary/Conclusion: Some of these target genes have reported as endosomal pathway associated protein and shown the up-regulation in cancer cells. These findings suggest that the identification of target genes of these miRNAs provides the new insight into the cancer cell communication with the microenvironmental cells, which leads to a promising therapeutic approach against cancer progression.

PF07.04

Ras Tumour microvesicles biogenesis and signalling in drosophila

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Introduction: Tumour-derived exosomes and microvesicles are increasingly implicated in cancers. Their respective functional contributions to cancer progression and the related mechanisms remain poorly defined. This is partly because current techniques, centered on differential centrifugation, do not permit adequate and specific isolation of pure exosomes or MV for targeted functional studies. More importantly, the paucity of animal models to address mechanistic and functional questions in tissues has further limited our knowledge on the role of extracellular vesicles in cancer biology

Methods: Using a Drosophila Ras tumour model, we have identified a strategy to specifically label and genetically manipulate tumour microvesicles in tissues for mechanistic studies.

Results: We will discuss some of our preliminary results on the dynamic of microvesicle biogenesis and their role in Ras tumour-macrophage signalling interaction.

Summary/Conclusion: Together with the power of Drosophila genetics, this in vivo system will enable novel insights into microvesicle biogenesis and function during tumour progression.

PF07.05

Src in endosomal membranes promotes exosome secretion and cancer progression

Chitose Oneyama

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Introduction: c-Src is a membrane-associated tyrosine kinase that has key roles in the signalling transduction

that controls cell growth, adhesion and migration. In the early stage of carcinogenesis, c-Src is activated under the plasma membrane and transduces oncogenic signals. Previous reports demonstrate that c-Src is localized to intracellular membranes, such as those of endosomes. However, the functional significance of endosomal c-Src in cancer is not well understood.
Methods: We examined intracellular localization of active c-Src, and in intermediate sections we found c-Src localized in perinuclear regions. In co-localization experiments with organelle markers in Src-transformed cells, active c-Src was present with the late endosome markers, including CD9 and CD63, which are also known as canonical exosome markers. We examined exosome secretion in c-Src-transformed cells.
Results: Our results indicate that activated c-Src in the endosomal membrane promoted the secretion of exosomes, in which c-Src was encapsulated. In addition, the ESCRT-interacting molecule, Alix was identified as a c-Src–interacting protein in exosomes. We revealed that the interaction between the SH3 domain of c-Src and the proline-rich region of Alix activates ESCRT-mediated intra-luminal vesicle (ILV) formation, resulting in the upregulation of exosome secretion in c-Src–transformed cells. We observed also a correlation between malignant phenotypes and Alix–dependent aberrant exosome secretion in c-Src–upregulated cancer cells.
Summary/Conclusion: Our findings indicate that c-Src-mediated activation of Alix promotes ILV formation in MVB, resulting in increased exosome secretion from various human cancer cells with activated c-Src. These data suggest that dysfunctions of exosome secretion suppress cell transformation, offering a novel signalling target and strategy for cancer therapeutics.
Funding: JST, PRESTO Grant Number JP1005457, Japan.

PF07.06

Modulation of Sphingosine-1-phosphate lyase and its implication in release of apoptotic exosome-like vesicle
Jihyo Kim, Jaehark Hur and Yong Joon Chwae

Introduction: Biogenesis of apoptotic exosome-like vesicles (AEVs), which can function as damage-associated molecular patterns, is reported to be regulated by sphingosine-1-phosphate (S1P)/S1P receptor 1/3 signalling. Thus, cellular S1P levels could be key factors in the biogenesis of AEVs. As is well-known, S1P is synthesized from sphingosine by sphingosine kinase 1/2-mediated phosphorylation and irreversibly degraded into fatty aldehydes and phosphoethanolamine by the enzyme, S1P lyase. In the present work, we investigated the role of S1P lyase in biogenesis of the AEVs and its molecular modulation in the apoptotic processes.
Methods: Preparation of AEVs: The conditioned medium was centrifuged for 10 min at 200 × g and twice for 20 min at 2,000 × g to remove cellular debris and apoptotic bodies. The pellets were collected by overnight incubation in 8% PEG6000 and 0.5 M NaCl, and washed by ultracentrifugation at 100,000 × g for 70 min.
Results: S1P lyase was degraded caspases-dependently in HeLa cells by apoptotic stimuli. Over-expression of N-terminal 3X flag- and C-terminal HA-tagged S1P lyase turned out that C-terminal region of S1P lyase was degraded. However, S1P lyase was not a direct target of caspases because mutations of Asp residues at C-terminal regions did not block its degradation. Possibly, S1P lyase might be a substrate of calpain in that co-treatment of a calpain inhibitor, PD150606 with staurosporine inhibited the degradation of S1P lyase. In consistent with this, knock-down of an endogenous inhibitor of calpain, calpastatin increased the degradation of S1P lyase while knock-down of calpain small subunit, CAPNS1 decreased the degradation of S1P lyase. Functionally, mutant form of S1P lyase deleted in C-terminal 21 amino acids showed decreased enzyme activities as well as less inhibitory effect on release of the AEVs when compared with wild type.
Summary/Conclusion: C-terminal degradation of S1P lyase during apoptotic processes contribute to enhancement of biogenesis of the AEVs, possibly through decreasing enzymatic activities of S1P lyase and subsequent increment of S1P in ER region. Although degradation of S1P lyase is caspases-dependent, S1P is not a direct substrate of caspases. It would be probable that S1P lyase was degraded by calpain, activated caspase-dependently.

PF07.07

Super-repressor-IκB-loaded exosome improves survival in a mouse model of sepsis and attenuates sepsis-induced inflammation
Youngeun Kim[a], Hojun Choi[b], Amin Mirzaaghasi[b], Eunsoo Kim[c], Kyungsun Choi[c] and Chulhee Choi[c]

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Introduction: The nanoparticles referred as exosomes play an active role in intercellular communication. The ability of exosomes to travel between cells and deliver their cargo, which includes proteins and nucleic acids, makes them an appealing cell-free therapy option to treat multiple diseases. Super-repressor IκB (srIkB)

which is S32A and S36A mutant form of IκB can continuously inhibit NF-κB because it is not phosphorylated by IκB Kinase and degraded by proteasome. Therefore, it has the great potential as a treatment for various inflammatory diseases. 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 via reversible protein–protein interactions. Here, we generated opto-genetically engineered exosome system to load srIkB into newly generated exosomes. Treatment with srIkB-loaded exosomes significantly reduced tumour necrosis factor-α-induced translocation and DNA binding of the p65, a subunit of NF-κB, in HeLa cells. Furthermore, srIkB-loaded exosomes administration improved survival in the cecal ligation and puncture (CLP)-induced sepsis mouse model and attenuated lipopolysaccharide (LPS)-induced systemic inflammation. In addition, in sepsis-induced mice, exosomes accumulated in the spleen and liver after intraperitoneal injection. This finding may be helpful for understanding the mechanism about how the administration of srIkB-loaded exosomes facilitates the recovery from sepsis. Taken together, these results show that srIkB-loaded exosomes could potentially be a novel anti-inflammatory and immunosuppressive cure in the treatment of sepsis and septic shock.

Methods: ABC

Results: ABC

Summary/Conclusion: ABC

PF07.08

Efficient delivery of Glucocerebrosidase Lysosomal Enzyme via EXPLOR technology for treatment of Gaucher's disease

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Introduction: Many intracellular proteins with great potential as biopharmaceutical drugs have been identified. However, numerous challenges associated with intracellular protein delivery have yet to be solved. Over the past years, extracellular vesicles including exosome have been regarded as a new paradigm for soluble protein delivery into cells or tissues. Because of their biological functions and features, exosomes are expected to be a novel treatment for diverse diseases, such as cancer and rare genetic disorder diseases. 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 via reversible protein–protein interactions. Here, we demonstrate the intracellular delivery of β -glucocerebrosidase (GBA) as functional proteins from the exosomes to the target cells. We generated opto-genetically engineered exosome system to load GBA, which is an enzyme deficient in Gaucher disease patients, into newly generated exosomes. Treatment with GBA-loaded exosomes showed the significant increase of intracellular levels of cargo proteins and their function in recipient cells in both time- and dose-dependent manner. In the present study, we tested lysosomal localization of GBA-loaded exosome in the target cells and compared the the efficacy with an analogue of the human GBA, VPRIV, to suggest it as a potential drug candidate in Gaucher disease.

Methods: ABC

Results: ABC

Summary/Conclusion: ABC

PF07.09

Sequence-specific release of EV-associated RNAs

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Introduction: Extracellular vesicles (EVs) contain different classes of RNAs, such as mRNA, miRNAs and circRNAs. As shown for miRNA and circular RNAs, RNAs are selectively exported into vesicles [1–2]. However, the factors or mechanisms that contribute to this specificity remain elusive. Thus, for example, a so-called Exo-motif has been described for miRNAs, which, however, cannot be transferred to all miRNAs classes, and for circRNAs a possible size-dependent export was suggested. In addition, only a few putative protein factors involved in packaging have been described [2].

Methods: To determine the export signals for the selective release of certain RNA species into EVs, we designed a modified in vivo SELEX approach (Systematic Evolution of Ligands by Exponential Enrichment) for identifying putative RNA sequence elements. We generated a random sequence pool (N40), which was transfected and expressed into HEK293 and HeLa cells. Moreover, several expression constructs were used, which consist of either an RNA Pol II or a Pol III promoter to analyze possible modification effects on the 5'-end of the RNA. Similarly, we introduced transcription terminators at the 3'-end to

prevent possible polyadenylation. EVs were isolated, followed by RNA isolation, library preparation, RNA-seq analysis and bioinformatic identification of enriched RNA motifs.

Results: We developed a new SELEX-based approach to identify enriched sequence motifs within EV-RNAs. For this, we have generated constructs that express long degenerate sequences but are still relatively small in total (85 nts). In a first attempt, we analysed the expression of the degenerated sequences and were able to recover these sequences from EV-RNAs. Detailed sequence and motif enrichment analyses are now in progress.

Summary/Conclusion: Here we described a novel approach to determine specific sequence motifs required for selective loading of RNA into EVs. This unbiased method should contribute to our understanding of how RNAs are specifically packaged into EVs.

References: [1] Preußer et al. 2018, J Extracell Vesicles.; [2] Villarroya-Beltri et al. 2013, Nat Commun.

PF07.10=OWP2.14

Isolation of extracellular vesicles from extracellular matrix based hydrogel 3D cell cultures

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Introduction: Cancer-derived extracellular vesicles (EVs) are commonly studied and isolated from two-dimensional (2D) cell cultures. Nevertheless, three-dimensional (3D) culture systems with extracellular matrix (ECM) provide physiologically more relevant system to mimic *in vivo* tumour growth and progression of invasion. However, there are currently no methods to efficiently isolate EVs from ECM-based 3D cultures. For that purpose, we established a protocol for isolating EVs from cancer cells growing in a 3D ECM-based hydrogel.

Methods: Human prostate cancer PC3 cells were grown in 3D to form spheroids in a commercially available ECM-based hydrogel and the growth media was collected every two days for a period of 14 days, during which the spheroids grew invasive. The respective media were differentially centrifuged at 2K, 10K and 100K g and the pellets were resuspended in PBS. The EVs were analysed by western blotting (WB) against the common EV markers CD81, CD63 and CD9.

Results: Our preliminary data shows a step-wise increase of the EV markers in the media as the PC3 spheroids formed, expanded and invaded to the surrounding 3D ECM. The EVs produced by non-invasive or invasive spheroids are currently being characterized with nano tracking analysis, electron microscopy and WB.

Summary/conclusion: This study demonstrates that EVs can be isolated from 3D ECM-based hydrogel cell cultures, which recapitulate the tissue architecture of solid tumours. Our results suggest that 3D cancer cell cultures have dynamic EV secretion determined by the phenotype of the spheroids. Taken together, we present a novel protocol for EV isolation from a 3D culture system and provide a platform to investigate EVs from *in vivo* mimicking conditions.

Funding: This project is funded by Magnus Ehrnrooth Foundation, K. Albin Johansson Foundation and Åbo Akademi University.

PF08: EVs in Tissue Injury and Repair
Chairs: Johannes Grillari; Bas van Balkom
Location: Level 3, Hall A

15:30–16:30

PF08.01

The role of adipocyte-derived extracellular vesicles in vimentin mediated fibrosis

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Introduction: Vimentin is involved in wound healing by mediating fibroblast proliferation, epithelial-mesenchymal transition processing, and collagen accumulation, but its functional contribution to this process is not clear. Adipocyte-derived extracellular vesicles (EVs) have the potential to promote wound healing by controlling molecular processes on recipient cells.
Methods: Differential centrifugation was used for the isolation of EVs from wilde-type (WT) and vimentin knockout 3T3lt1 (VIM-/-) cells. Electron microscopy and Western blot were used to characterize EVs from the cell culture media. In vitro analysis of cell migration and proliferation were performed using wound scratch assay and cell-derived matrices (CDM) in response to WT and VIM-/- EVs.
Results: Our preliminary data shows that Human Dermal Fibroblasts (HDF) show different significant migration responses in the presence of Adipose-derived Stem Cells (ASCs) EVs. Especially, exosomes treatment induced more migration compared to other EVs. Also, vimentin affected the results of the exosome treatment in fibroblast proliferation and migration assays.
Summary/Conclusion: This study is expected to reveal novel mechanisms of ASCs-derived exosomes regulating fibroblast activities in physiological wound healing and fibrosis.
Funding: This project was funded by Sigrid Juselius Stiftelse and Åbo Akademi University.

PF08.02

Effect of exosomes from human adipose-derived stem cells on hair growth

Hye-In Choi[a], Eun Wook Choi[b], Sang Won Yoon[b] and Byung-Soon Park[b]

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Introduction: Hair loss (alopecia) is a common medical problem affecting both men and women, and is caused by genetics, hormonal changes, medical condition or medications. Adipose-derived stem cells (ASCs) have been reported as an important component of regenerative medicine and cell therapy for hair loss. Recent studies have demonstrated that exosomes from mesenchymal stem cells and DPCs may regulate the hair follicle development and hair growth. In this study, we investigated the function of ASC secreted exosomes (ASC-exos) on hair growth.
Methods: Exosomes were isolated from the conditioned medium of human ASCs. The effects of ASC-exos on hDPC proliferation were evaluated using CCK-8 assays. The mRNA expression of growth factors was investigated using real-time PCR. Additionally, anagen induction was evaluated using an *in vivo* mice model. Furthermore, we analyzed the profile of exosomal microRNAs (exo-miRNAs) by microarray analysis, which was isolated from ASC-exos.
Results: We found that the ASC-exo enhanced the cell proliferation of DPCs. Also, quantitative real-time PCR showed that the expression of genes related with hair growth, such as transforming growth factor β-2, noggin, was increased after being treated with ASC-exos. Additionally, ASC-exos treatment accelerated the anagen hair induction when topically applied to C57BL/6 mice. Based on this finding, we conducted microRNA analysis and selected 12 miRNAs that contribute to regulation of hair growth.
Summary/Conclusion: Our results show that the exosomes from ASCs have a potential to activate DPCs and promotes hair growth in vivo and may use in treatment of hair loss.

PF08.03

Paracrine regenerative function of mesenchymal stem cells is not affected by chronic kidney disease

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Introduction: Cell-based therapies have been developed to meet the need for curative therapy in chronic kidney disease (CKD). Mesenchymal stromal cells (MSCs) enhance tissue repair and induce neoangiogenesis through paracrine action of secreted proteins and extracellular vesicles (EVs). Administration of allogeneic MSCs is less desirable in a patient population likely to require a kidney transplant, but potency of autologous MSCs should be confirmed, given previous indications that CKD-included dysfunction is present. While the immunomodulatory capacity of CKD MSCs has been established, it is unknown whether CKD affects wound healing and angiogenic potential of MSC-derived CM and EVs.

Methods: MSCs were cultured from BM obtained from kidney transplant recipients (N = 15) or kidney donors (N = 17). Passage 3 MSCs were used for experiments and collection of conditioned medium (CM). EVs were isolated from passage 8 MSCs from 13 male participants. *In vitro* pro-migratory and pro-angiogenic capacity of bone marrow (BM) MSC-derived CM and EVs was assessed using an *in vitro* scratch wound assay and Matrigel angiogenesis assay. Our methods are in agreement with the declaration of Helsinki and we obtained written consent from bone marrow donors.

Results: Healthy and CKD MSCs exhibited similar differentiation capacity, proliferation and senescence-associated β-galactosidase activity. Scratch wound migration was not significantly different between healthy and CKD MSCs (p = 0.18). Healthy and CKD CM induced similar tubule formation (p = 0.21). There was also no difference in paracrine regenerative function of EVs (tubulogenesis: P = 0.46; scratch wound: P = 0.6).

Summary/Conclusion: Our results indicate that CKD does not affect the regenerative potential of CM and EVs derived from CKD BM MSCs. This suggests that autologous MSC-based therapy is a viable option in CKD.

Funding: Netherlands Organisation for Scientific Research (NWO)

PF08.04

Cell to cell interactions orchestrated by exosomal MiRNAs between pathogenic- and Non-pathogenic corneal endothelial cells

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Introduction: Corneal endothelial dysfunction such as bullous keratopathy (BK), Fuchs' endothelial corneal dystrophy (FECD) can be restored only with corneal transplantation. We have recently developed a cell-injection therapy using cultured human corneal endothelial cells (cHCECs) (New Eng J Med.2018). Cultured HCECs have an inclination towards cell-state transition (CST). The expression of miRNAs is essential in the regulation of many cellular processes closely linked to CST in cHCECs. Here, we studied the role of exosomal miRs in pathogenesis of BK and FECD.

Methods: The composition of heterogeneous cHCEC subpopulations (SPs) were verified in regard to their surface cluster determinant (CD) markers. The profiles of miRs in cells, culture supernatants (CS) and in fresh corneal tissues were detected by 3D-Gene® Human miRNA Oligo chip (Toray). Exosome surface markers were measured either directly by Exo Screen or by WB after ultracentrifugation. PKH-labelled exosome was applied for the evaluation of the incorporated exosomes in cHCECs with distinct CD44 expression levels.

Results: MiR34a-5p and miR-378 family were detected only intracellularly and were strikingly lowered in pathogenic corneal endothelium. Candidate miRs in CS to discriminate CD44- SPs from those with CD44 ++~+++ phenotypes were miRs 23a-3p, 24-3p, 184, 1246, 1273 and 1285-3p. Among these miRs 23a-3p, 24-3p and 184 have a tendency to decrease in senescence-disposed cHCECs, the inversely correlated decrease with upregulated CD44. It is of note that lowered expression of cellular miR-378 induced the elevated gene expression of IL-8, MCP-1 and VEGF, and the increased secretion of exosomal miRs 23a-3p / 24-3p / 184 / 1273e / 1285-3p. CD9+ exosomes were more elevated in cHCEC CS with senescence-like CST than those without CST, indicating the possible import of these extracellular vesicles into cHCECs without CST. Compared with non-CST, CST cHCECs have a tendency to incorporate more exosomes.

Summary/Conclusion: MiRNAs in exosomes serve as an alternative tool to qualify cHCEC SPs. In this current study, we present the first finding that the lowered miRs in pathogenic tissues may induce the groups of exosomal miRs reliant on the depolarized CD44++~++ + HCECs.

PF08.05

Urinary CRK1 positive vesicles yield novel insight into microvesicular signaling of the kidney

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Introduction: While specific functions of microvesicles have been uncovered in many fields of biology and medicine, very little is known about their role in kidney health and disease. Recently, a new subgroup of microvesicles was discovered in human and murine cell culture as well as a model of glomerulonephritis. These vesicles are shed upon apoptosis and trigger proliferation in neighbouring cells, hence named apoptotic compensatory proliferative signalling vesicles – ACPSVs. As these vesicles could be isolated from kidney tissue, we hypothesized that a fraction is shed into the urine and can be isolated for further analyses.
Methods: We established a protocol of differential centrifugation and filtration to isolate ACPSVs from urine samples of healthy control subjects and patients suffering from different nephropathies. With western blot analysis and immunofluorescence microscopy, we validated the presence of ACPSVs and investigated the cellular origin of the vesicles. Whole lipid quantification was used to determine vesicle amount and to normalize the protein content. To identify the potential of initiating proliferation, HeLa cells were counted 24 h after treatment with freshly isolated urinary vesicles.
Results: The employed protocol lead to a robust isolation of spherical vesicles ranging between 0.6–1.8 μm containing the ACPSV marker protein CRK1. Further protein analysis revealed the presence of Podocin and Nephrin, pointing to a clear podocyte origin of a fraction of these vesicles. Similar results could be obtained for vesicles originating from the proximal tubulus and the collecting duct.
Summary/Conclusion: Our study represents the first analysis of urinary CRK1 containing vesicles. Taken into account the presence of podocyte marker proteins in the vesicle fraction isolated, we hypothesize, that these are not only shed upon apoptosis, hence would not call the isolated fraction urinary ACPSVs. Ongoing studies aim to validate the potential to initiate proliferation on different renal cell types, to further identify the cellular origin as well as to determine differences in their function and content in the state of renal diseases. As these vesicles can be easily isolated in a high purity, they also represent a valuable source for biomarker research in various nephropathies.

PF08.06

Human adipose stem cells-derived vesicles improve pain and reduce cartilage destruction in an osteoarthritis rat model

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Introduction: Human mesenchymal stem cells (hMSC) release extracellular vesicles (EV) containing various proteins and RNAs, which can act as regulatory signals between cells. hMSC-EVs also have provided significant beneficial effects in various disease models. However, most mammalian cells secret small amount of EV, which is a limitation for development of therapeutics. Therefore, the next generation of EV-mimetic vesicles produced by serial extrusion of cells produces higher number of vesicles, and may be easier to scale up for therapeutic developments. In this study we aimed to test the efficacy of EV-mimetic vesicles derived from human adipose-derived stem cells (hASCs) on rat osteoarthritis (OA) model.
Methods: hASC-derived EV-mimetic vesicles (CDV) were produced by serial extrusions of cells through filters. The CDVs were characterized by transmission electron microscopy (TEM), nanoparticle analysis system (NTA), and western blot and flow cytometry. CDVs were injected into the joints in a MIA-induced osteoarthritis (OA) rat model. Improvement of pain after CDV injections was assessed by paw withdrawal threshold and weight bearing, whereas the joint destruction was evaluated by histology. We also estimated the effects of CDV on proliferation and migration of human chondrocytes in vitro by cell-counting and scratch assays.
Results: The CDV were 50–150 nm in diameter and carried multiple EV-associated tetraspanins (CD63, CD9, CD81). CDV-treated OA mice had reduced paw withdrawal and was more weight bearing 17 days after treatment than PBS-treated. Further, histology showed reduced joint defects at 24 days. CDV-treated OA models displayed significant improvement in paw

withdrawal behaviour and weight bearing analysis. Similarly, chondrocyte migration and proliferation were enhanced by CDV in a dose-dependent manner.
Summary/Conclusion: This study demonstrates for the first time the efficacy of hASC EV-mimetic vesicles in OA model. Most interestingly we have confirmed that hASC EV-mimetic vesicles can improve pain and regenerate defected cartilage. These results support the concept that a potential application of hASC EV-mimetic is osteoarthritis, by giving CDV locally into affected joints.

PF08.07

Natural and synthetic biomaterial mediated delivery of Mesenchymal Stem Cell derived exosomes

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Introduction: Mesenchymal stem cell (MSC) derived exosomes are versatile agents that possess immunomodulatory and regenerative properties. However, systemic delivery of natural or engineered MSC exosomes lacks site-specificity and can trigger ectopic effects. Therefore, biomaterial-mediated site-specific delivery of exosomes is important. As exosomal membranes are subsets of the plasma membrane. We hypothesized that MSC exosomes can bound to extracellular matrix proteins and the property can be used as a delivery technique.
Methods: To test this hypothesis, we evaluated the binding and delivery kinetics of MSC exosomes to and from fibronectin, type I collagen and their derivative peptides followed by *in vitro* and *in vivo* evaluation of their efficiency when delivered using this approach.
Results: Results indicated that MSC exosomes bound dose-dependently and saturably to fibronectin, type I collagen and their derivative peptides in an integrin mediated fashion. The presence of integrins on the exosomal membrane was verified by immuno electron microscopy and immunoblotting. Finally, exosomes bound to 3D hydrogels containing these motifs were able to promote differentiation of naive MSC *in vitro* and bone regeneration in a valvaria defect model *in vivo*.
Summary/Conclusion: Overall, this study shows that MSC exosomes can be tethered to natural and synthetic biomaterials for site-specific delivery to aid repair and regeneration of tissues.

Funding: This project is sponsored by NIH grant R01DE027404 and The Osteology Foundation Advanced Researcher award.

PF08.08

Exosomes secreted during chondrogenic differentiation of human adipose-derived stem cells for osteoarthritis treatment

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Introduction: Osteoarthritis (OA) is a chronic degenerative joint disease and the most common form of arthritis. Most of the current treatments focus on pain management and treatment options for repair and regeneration of damaged articular cartilage are limited. In recent years, stem cell-derived exosomes have been the spotlight as a therapeutic candidate due to their regenerative and immunomodulatory capabilities. In this study, we hypothesized that exosomes (Chondro-EXOs) secreted during chondrogenic differentiation of human adipose-derived stem cells (hASCs) may contain specific biochemical cues that promote the regeneration of damaged cartilage in OA animal model.
Methods: Chondro-EXOs were isolated from conditioned media during chondrogenic differentiation by pre-filtration in 0.2 μm, followed by tangential flow filtration (TFF) system (300 kDa MWCO). The isolated Chondro-EXOs were characterized using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), flow cytometry, western blot, and cytokine arrays. To evaluate the therapeutic efficacy of Chon-EXO, we injected a mixture of Chondro-EXOs (1×10^8 particles) and hyaluronic acid hydrogel (1%) once a week for 3 weeks at intra-articular site of MIA-induced subacute OA models. Knee joints were harvested at four weeks after MIA injection and analysed histologically by safranin O-fast green and haematoxylin and eosin (H&E).
Results: Chondro-EXOs were approximately 50-120 nm in diameter and expressed exosomal markers such as CD9, CD63, and CD81. Various soluble factors related to anti-inflammatory and cartilage regeneration were contained in Chondro-EXOs. *In vivo* studies demonstrated that Chondro-EXOs significant prevented proteoglycan degradation and attenuated the cartilage destruction in the damaged articular cartilage.
Summary/Conclusion: Our findings suggest that Chondro-EXOs act as a biological cue for cartilage

repair and provide a new therapeutic approach for osteoarthritis treatment.

PF08.09

hucMSC exosomes delayed diabetic kidney diseases by transported kinase ubiquitin system promoted YAP ubiquitination degradation

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Introduction: Diabetes mellitus (DM) is a type of metabolic disease. Diabetic kidney disease (DKD) is the important microvascular complications of DM, the leading cause of end-stage renal disease (ESRD). Human umbilical cord mesenchymal stem cell exosomes (hucMSC-Exosomes) can participated in a variety of tissue damage repair. In this study, we demonstrated that the mechanism which hucMSC-Exosomes delayed the progression of DKD.
Methods: The DKD rat model established by 45% high-fat diet combined with streptozotocin (STZ, 35 mg/kg,iv). DKD group (n = 12) and hucMSC-exosomes group (n = 12), control group (n = 6). Blood glucose, body weight and 24 h urinary albumin clearance were measured at 16 and 24 weeks. HE, PAS staining used to observed pathological of renal tissue, Sirius red staining to detected renal interstitial fibrosis. YAP protein in renal tissues with time. Confocal microscopy observed YAP in cytoplasm and nucleus location. The CO-IP showed that the ubiquitin bound by YAP protein was significantly increased. LC-MS/MS and west bolt confirmed CK1δ/β-TRCP existed in the exospores. Used the adenovirus shRNA experiment knockdown CK1δ/β-TRCP.
Results: hucMSC-exosomes can migrated to renal injury site and regulated blood glucose in tissues. hucMSC-exosomes intervention delayed the progression of DKD. Maintained rat weight, reduced serum urea nitrogen, the degree of interstitial fibrosis significantly weakened. Sustained high glucose stimulated activation of YAP. The YAP increased significantly with time which increased degree of interstitial fibrosis. hucMSC-exosomes transported CK1δ/β-TRCP repaired kinase ubiquitin system imbalance inhibited YAP activity that attenuated interstitial fibrosis of DKD. Our experiments confirmed that hucMSC-exosomes carried CK1δ/β-TRCP promoted YAP ubiquitination degradation.
Summary/Conclusion: hucMSC exosomes delayed diabetic kidney diseases by transported CK1δ/β-TRCP promoted YAP ubiquitination degradation reduced renal interstitial fibrosis.

Funding: National Natural Science Foundation of China: (81871496)

Zhenjiang Key Laboratory of Exosomes Foundation and Transformation Application High-tech Research, China: (ss2018003)

PF08.11

Neutrophil extracellular vesicles protect from joint breakdown in inflammatory arthritis

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Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune, inflammatory disease. Recently our understanding of the inflammatory component has progressed tremendously, however, even after the control of inflammation, joint damage, in particular cartilage breakdown, continues to progress leading to secondary osteoarthritis and patient disability. Extracellular vesicles (EVs), with their roles in cell-to-cell communication, present a novel opportunity for treatment within difficult to target joint tissues like cartilage. Neutrophil EVs are remarkable in their bioactions and are abundant within the joints of RA patients. Here we report the role of Neutrophil EVs in RA and their effect on cartilage breakdown.
Methods: EVs were generated from human neutrophils stimulated with TNF (20 ng/ml; 20 min), and tested in the K/BxN murine model of inflammatory arthritis.
Results: In murine inflammatory arthritis, intra-articular injection of neutrophil EVs (30–300x10^3 per joint), reduced knee swelling and displayed cartilage protective effects, measured as reduced loss of proteoglycans and improved structural integrity in the treated joints. Cartilage in EV-treated joints also maintained a higher content of Collagen type2, an important component of healthy cartilage, and contained fewer hypertrophic chondrocytes, abundant in diseased cartilage. Of great translational importance, this effect lasted at least 28 days, suggesting that administration of these EVs enacted positive circuits of protection characterized by a phenotypic change within the tissue, resulting in long lasting protective effects even after the EVs themselves have been cleared. *In vitro*, neutrophil EVs inhibited IL-1-induced cartilage breakdown and restored basal expression of cartilage specific genes.

Summary/Conclusion: Neutrophil EVs exert powerful and long lasting protective bioactions in inflammatory arthritis, modulating the ongoing joint inflammation while also protecting from cartilage breakdown.

Funding: Medical Research Council (MRC) Regenerative medicine research grant

PF08.12

Role of small extracellular vesicles in ageing

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Introduction: Ageing is a major risk factor for many human diseases. It is a complex process that progressively compromises most of the biological functions of the organisms, resulting in an increased susceptibility to disease and death. Hutchinson-Gilford progeria syndrome (HGPS) and normal aging share many cellular phenotypes: abnormal nuclear shape, dysregulated of epigenetic markers, increased DNA damage. Remarkably, partial reprogramming extended the lifespan of the progeric mice with remodelling of the epigenetic markers. The alteration in intercellular communication with age has been demonstrated to be due to senescent cells developing a senescence-associated secretory phenotype (SASP).

Methods: In this study, we have a characterization of small extracellular vesicles (sEVs) using *in vitro* normal and premature ageing models and the rejuvenation capacity of sEVs from young donors and iPSCs in old and progeria recipients.

Results: Firstly, we performed the evaluation of production of sEVs using Nanoparticle Tracking Analysis (NTA) and characterization of positive CD63/CD81 sEVs by flow cytometry. Then, we evaluated the rejuvenation potential of sEVs from young and iPSCs donors on old and progeria fibroblasts. We found an increment of sEVs production with the age and the capacity of sEVs from young and iPSCs donors to recover the proliferation capacity (BrdU) and epigenetic marker (H3K9me3) in fibroblasts from progeria and old donors.

Summary/Conclusion: These findings are important to the understanding about influence of the ageing on sEVs and the development sEV-based therapies in age-related diseases.

Funding: BBSRC (BB/P000223/1) and The Royal Society (RG170399) awarded to AOL. JFL and PCF are funded by the Xunta de Galicia Fellowships (Spain).

PF08.13

Rab27a dependent exosome secretion from tubular epithelial cell promotes albumin-induced tubulointerstitial inflammation

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Introduction: Tubular epithelial cells (TECs) secrete increasing exosomes under with proteinuric toxicity. However, the mechanism through which exosomes are produced and the effect on tubular cell haemostasis and tubulointerstitial inflammation are unknown.

Methods: Proteinuric renal disease model was induced by adriamycin (ADR) administration through tail vein. Urinary albumin was determined at 0, 7, 14, 21 and 23 days after ADR injection. For *in vitro* studies, TECs were treated with albumin. Exosomes were purified from isolated tubules of kidney and cell culture supernatant for characterization and functional study.

Results: Urinary albumin was significantly increased in ADR-treated mice 2 weeks after injection compared with controls. Exosome production was increased significantly in kidneys and tubules of ADR mice and in TECs with albumin exposure, confirmed by electron microscopy, western blotting analysis of exosome markers and EXOCET. Interestingly, we showed increasing levels of Rab27a mRNA and protein both in the tubules of ADR-injected mice and in BSA-treated TECs in a dose dependent manner. Furthermore, the increased exosome production was dependent on Rab27a up-regulation since silencing of Rab27a reversed the exosomes secretion. Importantly, albumin was present in TEC-derived exosomes after BSA exposure. Impressively, lysosomal degradation of albumin was increased while the mRNA expression of inflammatory cytokines was reduced after inhibition of exosome secretion by Rab27a silencing in TECs treated with BSA. To explore the effect of TEC exosome production under albumin exposure, TEC-exosomes were purified and added to naïve TEC. Up-regulation of inflammatory cytokines were found in receipt TECs. Lentivirus Rab27a-inhibitor intrarenal injection reversed tubulointerstitial inflammation and increased survival of ADR-induced mice through stably inhibiting Rab27a expression. Clinically, high levels of Rab27a were found in tubules and correlated with the magnitude of urinary exosomes in patients with chronic kidney disease.

Summary/Conclusion: These results suggest that Rab27a-dependent exosomes secretion drive albumin escaping degradation and secreting into extracellular fluid may exacerbate TECs injury by enhancing inflammatory response and consequently leading to tubulointerstitial inflammation.

PF09: Detection of EV-based Biomarkers
Chairs: Fabia Fricke; Shinichi Kano
Location: Level 3, Hall A

15:30–16:30

PF09.01

Extracellular vesicle (EV) extraction and characterisation in amniotic fluid (AF)

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Introduction: During pregnancy, placental-derived EVs have been identified in maternal blood and AF thus are implicated in cell-to-cell communication. We hypothesize that placental-derived EVs released in amniotic fluid may possess angio-modulating properties that could be relevant in placental angiogenesis and that these characteristics may be altered in pre-eclampsia (PE), a pregnancy complication characterised by hypertension and proteinuria causing neonatal morbidity and perinatal mortality.

Methods: The amniotic fluid was obtained from normal pregnancies during caesarean sections. The physiochemical characteristics were tested by Nanosight technology (NTA) and characterization of exosomal markers was carried through FACS using microspheres and MASPlex exosome kit. MASPlex kit simultaneously detects 37 exosome surface epitopes.

Results: We set up a method for EV isolation from AF based on subsequent dilution with PBS; first centrifugation at 10,000 g for 30 min at 4°C, filtration through a 0.45 µM filter and ultracentrifugation at 100,000 g for 2 h in 4°C. The averages EV concentration was 4.34×10^{11} particles/ml with a mean peak of 240.45 nm, measured by NTA. FACS analysis showed presence of angiogenic markers VEGFR 1,2,3 and CD105, immunological markers HLA ABC, HLA DR, exosome specific markers CD81 and CD63 also CD133, which indicates kidney origin. By using the MASPlex kit, we set up a semiquantitative method for detection of 37 different potential AF-EV surface markers in one sample simultaneously. We confirmed the heterogenic characteristics of AF-EVs, including expression of immune system markers CD209, CD62P, CD11c, CD20 and endothelial markers CD146 and CD41b.

Summary/Conclusion: The characterisation of the AF-EVs with NTA and FACS demonstrates the composition and size as well as presence of markers of different origin including kidney, immune system and endothelium. The investigation of EV properties in healthy and diseased placenta could prove useful in the future as a diagnostic tool to understand and diagnose pregnancy-associated diseases.

Funding: This work was supported by the iPlacenta project founded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 765274

PF09.02

Evaluation of non-invasive biomarkers for monitoring functional status of endometrium

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Introduction: Endometrium is a complex tissue with self-renewing properties, normally undergoing cyclic modifications regulated by ovarian steroids divided into proliferative and secretory phase. The transcriptomic profile of the endometrium is influenced by other endometrial cell types (glandular epithelial and stromal) in both physiological and pathological conditions. These cells have mutual paracrine effects partially mediated by EVs, and they grow in a cycle-dependent manner. To assess the endometrium status, several invasive or expensive techniques are currently employed, including immunohistochemistry (IHC) on tissue biopsy, cytology and imaging. Development of protocols for the isolation of EVs from novel biological sources is an extremely attractive means to surrogate endometrial biopsies. These novel protocols may enable the identification and sensitive detection of specific endometrial EV biomarkers for diagnostic solutions in reproductive medicine, endometriosis or cancer.

Methods: Samples: primary endometrial cultures, urine from healthy donors in secretory phase; Differential centrifugation, size exclusion chromatography (SEC),

immunobeads for EV isolation; Nanoparticle Tracking Analysis (NTA), BCA assay, ELISA, HS Qubit, ddPCR, SPR, FACS for EVs and EV markers quantification and characterization.

Results: We provide new evidence that urine is a surrogate biofluid suitable for the detection of endometrial EV biomarkers. Using pre-selected antibody panels, we identify specific endometrium EV binding antibodies in relevant *in vitro* models. Coupling immune-isolation to pre-analytical protocols for urine processing and sample quality testing enables detection of a panel of endometrial genes in urine-recovered EVs.

Summary/Conclusion: Overall, the study provides a tool for non-invasive monitoring of the functional status of the endometrium, supporting biomedical niches such as assisted fertilization and diagnosis of endometriosis.

Funding: ENDEvor POR Region Tuscany (identification of the project) and Exosomics R&D Programme

PF09.03

Unveiling autologous blood doping: comparative analysis of different purification strategies for urinary extracellular vesicles pioneering miRNA biomarker research

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Introduction: Autologous blood doping (ABD) increases the oxygen capacity via re-infusion of a person's autologous red blood cells. It is therefore used by endurance athletes with a high level of unreported cases, especially since reliable methods for unequivocal detection are still lacking. To support the Worldwide Anti-Doping Agency (WADA) in protecting the athletes' healthiness and ensuring harmonization, hereinafter extracellular vesicles (EVs) and their conveyed cargo were used as potential biomarkers. Since degraded red blood cells and their content are eliminated via the kidney and urine, the urinary EV population and their microRNA (miRNA) profile were particularly focused.

Methods: After study approval by the local ethics committee, written informed consent was obtained of 30 healthy men undergoing different ABD intensities and several sampling time points. Consistent compliance with the "Declaration of Helsinki" was assured. Due to a lack of standardization in urinary EV purification, five different isolation strategies were evaluated: ultracentrifugation, membrane affinity, spin column chromatography, immunoaffinity and precipitation. After EV characterization by nanoparticle tracking analysis, western blotting, and transmission electron microscopy, total RNA was isolated and a library for small RNA sequencing was prepared. The resultant successful strategy was then applied to all the collected samples which were equally analysed regarding their EV distribution and miRNA content.

Results: The comparative analysis disclosed huge discrepancies with respect to EV yield, population, and purity, as well as RNA yield and detected miRNAs. By applying the best performing strategy, which was based on immunoaffinity, significantly higher amounts of urinary EVs and several significantly differentially regulated miRNAs were observed after ABD.

Summary/Conclusion: Urinary EVs and their miRNA profile hold indeed promising attempts for the clear separation of ABD and non-doped athletes. Moreover, the included complex comparative methodological analysis contributes enormously to future standardization and comparability of urinary EV research.

Funding: The current project has been financially supported by the WADA.

PF09.04

Extracellular vesicles as graft biomarkers to address lung transplantation outcome

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Introduction: In the medical practice, lung transplantation is the last therapeutic option for end-stage pulmonary failure, when other treatments are no longer effective. Yet, only 15-20% of the multi-organ donors have suitable lungs. Furthermore, clinical complications may rise after organ retrieval following ischemia–reperfusion lung injury, such as primary grafts dysfunction or chronic lung allograft dysfunction. Currently, clinical parameters implemented to assess the quality of the graft have failed to evaluate tissue damage at the cellular level and to predict transplantation outcome. Therefore, we focused our attention on extracellular vesicles (EV) as innovative, non-invasive

biomarkers urgently needed to assess lung quality and monitor organ engraftment.

Methods: Research activities involving human subjects complied the Declaration of Helsinki. Informed consent and local ethics committee approval were obtained. Size and concentration analysis were performed by nanoparticle tracking analysis (Nanosight NS300, Malvern).

Results: Preliminary results showed the presence of EV of different sizes in bronchoalveolar lavage (BAL) and plasma of both donors and recipients. EV presented highly polydispersed size distributions in a 50–1000 nm range. Different EV production kinetics were observed in the recipients (10E08-10E10 particles/mL range): BAL samples showed concentration peaks within 72 h post-transplant and a subsequent decreasing trend, whereas plasma samples showed a slightly increasing trend. EV samples will be analysed for RNA content and antigen expression, and correlation with lung transplantation outcome will be evaluated at the conclusion of the follow-up.

Summary/Conclusion: The identification of specific EV kinetics patterns and RNA signatures represents a promising approach to define biomarkers useful for thoracic surgeons who want to manage in advance complications associated with lung transplantation.

PF09.05

Expression profile analysis of miRNAs in serumal exosomes as sensitive biomarkers before and after hematopoietic stem cell transplantation

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Introduction: To investigate the differential expression of miRNAs of candidate genes in peripheral blood serum of patients with acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT).

Methods: There were a total of 30 patients with aGVHD and without aGVHD within 100 days after allogeneic hematopoietic stem cell transplantation in leukaemia; serumal exosome and RNA were extracted before and after the transplantation; image acquisition and data analysis were performed after hybridization of gene chips; candidate genes associated with the occurrence of aGVHD were screened out based on the differential expression of miRNAs.

Results: The expression profile of miRNAs after hybridization of gene chips showed increasing or decreasing tendency of miRNAs before and after the transplantation. Compared with the control group, for the miRNAs whose signal fold multiples greater than 10 folds, there were 11 miRNAs increased and 26 decreased in the aGVHD group. The expression of hsa-miR-3976, hsa-miR-122-5p, hsa-miR-3125 were significantly up-regulated and the expression of hsa-miR-4687-5p, hsa-miR-941, hsa-miR-4769-5p were down-regulated; these six miRNAs were listed as candidate miRNA gene sensitive biomarkers in peripheral serum.

Summary/Conclusion: Through Go, pathway and target gene analysis, candidate genes participate in regulating water-soluble vitamin metabolism, mitochondrial apoptosis and other biological processes, regulating cell membrane and organelle synthesis. The specific mechanism will be further studied.

Funding: Natural Science Foundation of Guangdong Province (2015–2018)

Free application project of Shenzhen Science and Technology Innovation Committee (2017–2020)

PF09.07

Circulating cancer-associated extracellular vesicles as early detection and recurrence biomarkers for pancreatic ductal adenocarcinoma

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is usually found at an advanced stage, although diagnosis at an early stage is unequivocally associated with better long-term survival. Therefore, there is an urgent need to develop early detection methods to improve these outcomes. Extracellular vesicles (EVs) attract much attention as potential biomarker because tumour cells have been shown to release EVs into circulation which mirror their cellular origin. Detection of cancer-associated EVs in body fluids from patients could serve as a non-invasive liquid biopsy for diagnosis and monitoring of cancer. The main objective of this study is the identification and detection of PDAC-specific EVs in patient-derived serum.

Methods: Total EV proteins were purified from three different stages of PDAC (stage II, III and IV, each group: six patient sera pooled) and healthy donors. Mass spectrometry was performed with purified EV proteins, and biomarker candidates were validated by immunoblotting in PDAC patient blood samples.

Results: Proteomics analysis identified over 500 proteins in each pooled samples, and we identified nine membrane proteins, which were detected in only PDAC patients, not in healthy donors. We focused on two proteins and performed immunoblotting for the validation of potential biomarkers in three stages of PDAC blood samples. As a result, these proteins were detected exclusively in EVs of PDAC patient sera including stage II. Moreover, we analysed a total of 33 samples from 11 PDAC patients who performed surgery at three time points; before surgery, after surgery and recurrence as an early stage model. As a result, these proteins were detected in EVs derived from preoperative samples and recurrence samples.
Summary/Conclusion: This study using unique recurrence samples as an early stage model shows that the identified EV-associated proteins have potential as early detection makers and warrant further investigation.
Funding: This work was supported in part by a Grant-in-Aid from the Japan Science and Technology Agency (JST) through the Center of Open Innovation Network for Smart Health (COINS) and a Grant-in-Aid from the Japan Agency for Medical Research and Development (AMED) through Project for Cancer Research and Therapeutic Evolution (P-CREATE: JP18cm0106402).

PF09.08

Exosome-encapsulated miRNA in urine as a non-invasive biomarker for prostate cancer

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Introduction: Prostate cancer (PCa) is the most common malignant tumours in male urinary system. Novel and non-invasive biomarker with higher sensitivity and specificity for the diagnosis of PCa are urgently needed. Exosomal microRNAs in circulating fluids have recently been reported to augment diagnosis and management of certain diseases, including cancer. The purpose of this study is to explore the diagnostic value of urinary exosomal miRNAs for PCa.
Methods: A urinary exosomal microRNA expression profiling was performed by next-generation sequencing using urine samples. Then, candidate miRNAs were selected and validated by qRT-PCR in 3 cohorts consisting of PCa patients, healthy controls and patients with benign prostatic hyperplasia (BPH). Receiver operator characteristic (ROC) analysis was used to evaluate the diagnostic and prognostic value of urinary exosomal miRNA in PCa.
Results: Five candidate miRNAs were found by NGS. Significant downregulation of urinary exosomal miR-375 was observed in PCa patients comparing with healthy controls, while miR-451a, miR-486-3p and miR-486-5p were found significantly upregulated. However, no significant difference was found for miR-16-2-3p. The expression level of urinary exosomal miR-375 showed significant correlation with clinical stage and bone metastasis of the patients with PCa ($p < 0.05$). ROC analysis demonstrated that the urinary exosomal miR-375, miR-451a, miR-486-3p and miR-486-5p are able to differentiate PCa patients from healthy controls, with the AUC of 0.788, 0.757, 0.704 and 0.796, respectively. The urinary exosomal miR-375 was found superior in discriminating localized PCa from metastatic PCa, with an AUC of 0.806. Additionally, PCa patients can be distinguished from BPH patients by using a panel combining urinary exosomal miR-375 and miR-451a, with an AUC of 0.726.
Summary/Conclusion: These findings demonstrate that the urinary exosomal miRNA can serve as a novel and non-invasive biomarker for diagnosing and predicting the progression of PCa.
Funding: Shaanxi Health and Family Planning Commission Foundation Project (2016D020), Xi'an Science and Technology Bureau Foundation Project (2017121SF/YX015) and Shaanxi Natural Science Foundation Project (2018JQ8010).

PF09.09

Unlocking the secret of salivary exosomes derived from HPV-driven oropharyngeal cancer

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Introduction: There has been a significant rise in the incidence of oropharyngeal cancer (OPC) associated with high-risk human papillomavirus (HPV), predominantly HPV-16 infections in high-income countries, especially when compared to HPV-negative head and neck cancer (HNC). Growing evidence supports the concept that exosomes (30–150 nm) loaded with unique bio-components (DNA, RNA and protein) play a salient role in cancer development and

progression. However, the role of exosomes in saliva obtained from HPV-driven OPC is still far from clear.
Methods: Morphology and molecular features of exosomes derived from three different saliva sampling methods: unstimulated saliva, acid-stimulated saliva and salivary oral rinses were examined using transmission electron microscopy (TEM), nanoparticle tracking (NTA) and western blot analysis. HPV-16 DNA detection in salivary exosome was determined using qPCR method. Proteome profile of salivary exosomes derived from both cancer-free controls and HPV-driven OPC patients was characterized using mass spectrometry analysis.
Results: Here, we showed that unstimulated saliva had the greater abundance of exosomes when compared to the other saliva sampling methods. In fact, the three common exosome markers (CD9, CD63 and CD81) were higher in unstimulated saliva method. Nevertheless, no appreciable difference in exosome morphology was found among the three different sampling methods. Furthermore, only salivary exosome derived from HPV-driven OPC had a detectable level of HPV-16 DNA. Intriguingly, the proteomic signature of salivary exosome was significantly different between cancer-free controls and HPV-driven OPC.
Summary/Conclusion: Taken together, our results showed that unstimulated saliva is an optimum sampling method for exosome characterization. More importantly, the development of a low cost non-invasive saliva-based test (salivary exosomal DNA and protein) will offer an opportunity to detect HPV-driven OPC, thereby opening new avenues in the future for clinical and commercial translation.
Funding: N/A

PF09.12

Determination of the protein cargo of colon cancer tissue-derived extracellular vesicles

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Introduction: Colorectal cancer (CC) is the third most common cancer to affect both men and women, and the third-leading cause of cancer-related mortality. In order to be curable, CC has to be diagnosed and treated by surgery before the tumour cells have started to metastasize. In order to find CC at an early stage, more sensitive biomarkers need to be developed. Extracellular vesicles (EVs) have in the last decade been recognized as major players in cancer biology but it's only in recent years that isolation protocols have reached enough sophistication for a truly meaningful proteomic analysis.
Methods: Tumour and non-tumour tissue (approx. 10 cm from tumour) were excised from 10 CC patients. The tissue was sliced into approximately 1 mm 3 pieces and partially digested with DNase and Collagenase in cell culture medium for 30 min at 37°C. Digested tissue was filtered through a 70 μm filter to remove tissue pieces and large fragments. Vesicles were isolated from the media with an isolation process consisting of differential ultracentrifugation and density gradient floatation aimed at isolating EVs. Isolates were then lysed, tryptically digested and Tandem Mass Tag labelled before analysis by mass spectrometry. The samples, 20 in total, were analysed in two rounds as "Set 1" and "Set 2", with each set containing EVs derived from five tumour tissues and five non-tumour tissues.
Results: In total, approximately 4000 proteins were identified and quantified, with 2567 and 3742 proteins identified in Set 1 and Set 2 respectively with an overlap of 2271 proteins between the sets. Proteins which after a t-test had a p-value lower than 0.05 and a fold change of at least two were considered as being differently expressed in tumour tissue EVs as compared to normal tissue EVs. 299 and 592 such proteins were identified in Set 1 and Set 2 respectively, with 125 meeting these requirements in both Sets.
Summary/Conclusion: EVs isolated directly from the tumour tissue microenvironment differ in their protein cargo from that of EVs resident in equivalent non-tumour tissue. Proteins carried by tumour derived EVs could potentially play a role in tumour biology by mediating signalling to neighbouring cells. Furthermore, these differentially expressed proteins could potentially function as biomarker candidates.

PF09.13

Characterization of small extracellular vesicles secreted by dermal fibroblasts

Marijana Jevtić[a] and Sarah Hetrich[b]

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Introduction: Dermal fibroblasts play a key role in epidermal proliferation and differentiation. They communicate with other cell types, playing a crucial role in the regulation of the skin (patho)physiology. Extracellular vesicles (EVs) are small membrane-enclosed vesicles (30-150 nm) that are

released from all cell types into the extracellular space and represent an important mode of cell-to-cell communication. Emerging data indicate that they play key roles in many (patho)physiological processes. However, there is currently very little information about the content and the function of EVs from dermal fibroblasts. Therefore, we aimed to isolate and characterize EVs secreted by dermal fibroblasts.

Methods: Dermal fibroblasts were isolated from juvenile foreskin and cultivated in DMEM supplemented with 7.5% FBS and 2 mM L-glutamine. On day 5 of cultivation, dermal fibroblasts were washed with PBS and further cultured in EV-depleted medium for 24h before collecting the medium. To elucidate the characteristics of EVs, EVs were isolated from conditioned medium by several ultracentrifugation and filtration steps. To verify the presence of EVs, nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and flow cytometry (FACS) were performed on the dermal fibroblasts-derived EVs.

Results: With FACS analysis of dermal fibroblasts, we proved that more than 95% of the cells were alive in the culture, what provide that we isolated pure EVs released by live cells. NTA and TEM analyses proved the presence of EVs, cup-shaped structure and size smaller than 150 nm. With FACS analysis of EVs, we proved that EVs are enriched with cytosolic protein present in EVs, Tsg101.

Summary/Conclusion: Here we present characterization of EVs secreted by dermal fibroblasts in terms of size, shape and cytosolic proteins present in EVs. In next steps, we plan mass spectrometry of the proteome of dermal fibroblasts and EVs secreted by dermal fibroblasts. EVs are able to interact with cells located nearby or distantly and EVs can be a way for carrying information from cell to cell. These findings may lead to identification of new signalling pathways in between dermal fibroblasts and other cells present in the skin, what could help us to understand the regulation of the skin physiology.

Funding: S.H. acknowledges financial support by the German Research Foundation (DFG HE 7440/4–1).

PF10: Advances in EV separation and concentration
Chairs: Stacey Gifford; Fuquan Yang
Location: Level 3, Hall A

15:30–16:30

PF10.01

Efficient clearance of lipoproteins from anti-coagulated and native blood-derived products to yield pure extracellular vesicle preparations

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Introduction: Extracellular vesicles (EVs) increasingly gain focus in regenerative medicine for promoting tissue repair and alleviating inflammation. However, there are no standards for EV isolation from patient blood nor for quality assessment owing to lack of knowledge about active components or mechanisms of action. It is known that high, low and very low density lipoproteins (HDL, LDL, VLDL) as well as chylomicrons copurify with EVs during isolation from various body fluids including blood via ultracentrifugation (UC) or size exclusion chromatography (SEC). The aim of our study was to develop an isolation strategy to purify EVs from blood derived products which are already in clinical use. Therefore, we analysed EV preparations from citrate-anticoagulated platelet-rich plasma (CPRP) and hypACT™ serum.
Methods: Particle concentrations after UC, SEC or a combination of both were assessed via nanoparticle tracking analysis (NTA). EVs were labelled with annexin V (AnnV), CD63 as well as CD41 and analysed by flow cytometry (FC). LDL and HDL content was determined in EV preparations by labelling of Apolipoprotein A1 (ApoA1) and Apolipoprotein B100/48 (ApoB-100) by FC as well as detection via Western Blot. Presence of EVs was confirmed by cryo electron microscopy.
Results: NTA revealed 100-fold higher particle concentrations after SEC than after UC or UC+SEC in both, CPRP and hypACT(TM) serum. AnnV, CD63 as well as CD41 were detected on EVs via FC. It also revealed efficient clearance of ApoB-100 bearing particles by UC, while ApoA1-positive particles persisted. SEC alone removed ApoA1-positive particles, but failed to remove ApoB-100 bearing particles. The combination of enrichment via UC and purification via SEC enabled efficient clearance of both lipoprotein species (ApoA1 as well as ApoB-100). These findings are also supported by Western Blot analysis.
Summary/Conclusion: EV preparations are commonly contaminated with lipoproteins due to their similar size and density. The coupling of UC to separate EVs from lipoproteins by density and SEC to yield separation by size enabled efficient clearance of lipoproteins from CPRP or hypACT(TM) serum and obtaining pure EV preparations.
Funding: The work was funded by the Wissenschaftsfonds of Lower Austria (NÖ) together with the European Fund for Regional Development (EFRE).

PF10.02

Proteomic and Lipidomic Analysis of Extracellular Vesicles from Human Plasma and Urine Purified by Asymmetrical Flow Field-Flow Fractionation

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Introduction: Extracellular vesicles (EVs) are composed of lipid bilayer membranes and they are a group of heterogeneous, nano-sized structures vesicles enriched with nucleic acids, proteins and lipids. EVs can be released by normal and cancer cells to their surrounding environments and they are also found in diverse body fluids, including blood, urine, saliva, cerebrospinal fluid, breast milk, seminal fluid. EVs play many important roles in numerous physiological and pathological processes.

In recent years, various studies on EVs have been conducted in the clinical research. EVs are rich in disease related biomarkers, and can protect the wrapped parent cells derived materials due to their double layer membrane structures and target the specific cells or tissues. EVs have promising potential for diagnostic and therapeutic applications, and can serve as biomarkers and targeting drug delivery systems.

Omics studies of EVs have been used for the discovery of biomarkers. The isolation of EVs are the key step for the omics studies on EVs.
Methods: Field-flow fractionation (FFF) technique was first invented in 1966 by J. Calvin Giddings. FFF has

unique properties enabling separation and characterization of macromolecules, polymers, proteins, colloids, cells and vesicles from 1 nm to 100 m at high resolution. AF4 has been reported to purify EVs from the supernatant of cell culture. In this study, we have developed AF4 based mothed for isolation of EVs from human plasma and urine. The proteomic and lipidomic analysis was performed using LC-MS/MS.
Results: EVs in human plasma were isolated from HDL and LDL with good resolution by an optimized AF4 conditions. EVs in human urine were also isolated from the high abundant protein uromodulin by optimized AF4 conditions after treatment with DTT reduction. Transmission electron microscopy (TEM), SDS-PAGE, Western Blot, proteomics and lipidomics are further applied for the studies on purified EVs from human plasma and urine.
Summary/Conclusion: The results reveal that AF4-based separation method for EVs is of high reproducibility, purity, recovery and continuous preparation and separation ability. The specific proteins and lipids have been identified from human plasma and urine EVs compared with the whole components in human plasma and urine

PF10.03

A centrifugation model to predict the behaviour of tumour biomarkers in liquid biopsies

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Introduction: Biomarkers in blood of cancer patients include circulating tumour cells (CTCs), tumour-educated platelets (TEPs), tumour-derived extracellular vesicles (tdEVs), EV-associated miRNA (EV-miRNA), and circulating cell-free DNA (ccfDNA). Because the size and density of biomarkers differ, blood is centrifuged to isolate or concentrate the biomarker of interest. Here, we applied a model to predict the effect of centrifugation on the purity of a biomarker according to published protocols.
Methods: The model is based on the Stokes equation and was validated using polystyrene beads in buffer and plasma. Next, the model was applied to predict the biomarker behaviour during centrifugation. The result was expressed as recovery of CTCs, TEPs, tdEVs in three size ranges (1–8 µm, 0.2-1 µm, and 0.05–0.2 µm), EV-miRNA and ccfDNA.
Results: Bead recovery was predicted with errors <18%. Most notable cofounders are the 22% contamination of 1–8 µm tdEVs for TEPs, and 50–82% of tdEVs <200 nm for ccfDNA. Based on our model, none of the evaluated protocols produces a pure biomarker. Thus, care should be taken in interpretation of obtained results, as, for example, results from TEPs may originate from co-isolated large tdEVs, and ccfDNA may originate from DNA enclosed in tdEVs <1 µm.
Summary/Conclusion: The Stokes model can be applied to predict the behaviour of biomarkers – including EVs- during isolation or concentration to other body fluids, which may facilitate the comparison of such protocols in e.g. EV-TRACK, further standardization of protocols, and develop optimal biorepository conditions.
Funding: This work is supported by the Netherlands Organisation for Scientific Research – Domain Applied and Engineering Sciences (NOW-TTW), research programs VENI 13681 (Frank Coumans), Perspectief CANCER-ID 14198 (Linda Rikkert), and VENI 15924 (Edwin van der Pol).

PF10.04

Effects of lipoprotein destabilization on isolation and analysis of plasma-derived extracellular vesicles

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Introduction: Plasma is one of the most commonly used sources of EVs since it is easy to access and is extensively used in clinical research and diagnostics. Isolation of pure EVs from such a complex biofluid is hard to accomplish due to presence of many contaminants (lipoproteins, soluble proteins and protein aggregates) that affect downstream application. Here, we are exploring effects of plasma acidification on isolation, purification and detection of EVs, as stand-alone or combined with other pre-analytical steps: lipoprotein lipase (LPL) and low-density lipoprotein receptor (LDLR) treatment, in line with further purification and analytical methods.
Methods: Plasma preclearing and EV isolation: differential centrifugation, tangential flow filtration (TFF), size exclusion chromatography (SEC), enzyme-coupled and affinity magnetic beads.

Quantification and characterization of EVs: ELISA, NTA (Nanoparticle Tracking Analysis), BCA assay, Western Blot, total RNA extraction and quantification.
Results: Preliminary results reveal 3–5 fold increase of EV protein signal in EV-enriched SEC fractions after plasma acidification, although lipoprotein profile in same fractions, as well as NTA counts and protein content, stay mostly unchanged compared to normal pH (control) samples. Additional steps aimed at separation of lipoproteins from vesicles, after lipoprotein destabilization through combination of size focusing, enzymatic digestion and ligand specific-depletion/selection, are described.
Summary/Conclusion: Our experiments are addressing the issue of plasma EV purification in attempt to deplete lipoprotein particles using different preanalytical approaches. Acidification, along with LPL and LDLR incubation, hold potential for lipoprotein removal.
Funding: This research is part of TRAIN-EV project, funded by EU grant under the Horizon2020 Marie Sklodowska Curie Innovative Training Network (MSCA-ITN) programme.

PF10.05

The stability of placental extracellular vesicles in different short-term storage conditions

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Introduction: Extracellular vesicles (EVs) are attracting considerable attention from a wide range of researchers because of their signalling capacity of relevance to health and many diseases. EVs are classified to macro-, micro-, and nano-EVs based on their size and carry complex cargos of RNAs, protein, DNA and lipids that can change the behaviour of target cells. Given the unique characteristics of EVs and that they are challenging to isolate in large quantities for use in experiments especially *in vivo* experiments it is important to be able to store EVs and maintain their quality. In this study we began to investigate the stability of human placental EVs which were extruded from first trimester placentae.
Methods: EVs were isolated from first trimester placental explants (range from 8 to 12 weeks of gestation, n = 8) and separated into micro- and nano-EVs by differential centrifugation. EVs were then individually stored in PBS at room temperature, 4°C or −20oC for up to 2 weeks. The concentration and the size of each type of EVs were measured by Nanoparticle Tracking Analysis at day 0, day 3, day 7 and day 14.
Results: The concentration of micro-EVs or nano-EVs which were stored at 4oC or room temperature was not significantly different between days 0, 3, 7 or 14. In contrast, the concentration of micro-EVs which were stored at −20°C was significantly reduced at both days 7 (p = 0.001) and 14, compared with the concentration of micro-EVs at day 0. The concentration of nano-EVs stored at −20°C was significantly reduced at day 14 (p = 0.04), compared with the concentration of nano-EVs at day 0. In addition, there was no difference in the modal (or mean) size of either micro- or nano-EVs regardless of the storage conditions at any time point.
Summary/Conclusion: we found that, at least in terms of concentration and size, short/medium-term storage of placental EVs at 4°C or room temperature was preferable to freezing. Further work is required to determine optimal storage conditions to maintain EV function.

PF10.06

Only a portion of the T cell-released exosomes has a capacity to destruct mesenchymal tumour stroma

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Introduction: Exosomes (Exo) released from single cells have been thought to be diverse populations in membrane structures, membrane charges and bioactive substances. We have reported that CD8 + T cell Exo can deplete mesenchymal tumour stromal cells and suppress tumour invasion and metastasis (*Nat. Commun*. 9: 435, 2018). In this study, we examined the diversity of CD8 + T cell Exo and destruction of mesenchymal tumour stroma.
Methods: H-2Kd-restricted and mutated (m) ERK2 136–144 peptide-specific TCR gene-transfected DUC18 mice were used in this study. DUC18 splenocytes were cultured for 7 days with mERK2 peptide, and obtained culture supernatant (sup) was used as a source of CD8 + T cell (CTL) Exo. Ultrafiltration (UF) of DUC18 culture sup was performed by tangential flow filtration system (KrosFlo TIFF system) using mPES MidiKros Filter Modules (MWCO 500 kDa or 750 kDa: Spectrum) at the entrance flow rate of approximately 50 mL/min. DEAE-sepharose Fast Flow (GE) was used as a carrier of cationic ion-exchange chromatography. DEAE-sepharose column (bed volume 8 cm^3) was equilibrated with 10 mM

Tris-HCl (pH7.5) containing 0.15 M NaCl. DUC18 Exo concentrated with UF was loaded on the column, and washed with TBS at over three column volumes. Exo bound with DEAE-sepharose were eluted by linear gradient of NaCl.
Results: By UF with 750 kDa MWCO mPES filter, CD8 + T cell Exo can be effectively concentrated more than 20 times without leaking. The concentrated CD8 + T cell Exo was adsorbed on a DEAE column and eluted with NaCl gradient of 0.15 M to 0.8 M. As a result, the various Exo fractions could be obtained from the difference of the levels of CD9 expression, CD90 expression, Granzyme B content, the Tsg101 content, and engulfment by mesenchymal stem cells. Interestingly, capacity of destruction of mesenchymal stroma was found only in Exo fraction eluted about 0.25 M NaCl, indicating that a part of CD8 + T cell Exo exerts a biological function.
Summary/Conclusion: We establish a novel method for Exo preparation according to the negative charge. Exo released from single cells are diverse populations with different physical properties, some of which exhibit biological significance.
Funding: This work was supported by a grant from the JST CREST (JPMJCR17H2).

PF10.07

Evaluation of the effects of acidification on isolation of extracellular vesicles from bovine milk

Md. Matiur Rahman[a], Kaori Shimizu[b], Marika Yamauchi[c], Ayaka Okada[b] and Yasuo Inoshima[b]

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Introduction: Acidification has shown potential for separating casein from raw bovine milk to facilitate isolation and purification of extracellular vesicles (EVs). The purpose of this study was to evaluate the effects of different acidification treatments on the yield and surface marker proteins of EVs from raw bovine milk.
Methods: Fresh raw bulk milk was collected from healthy dairy cows. Casein was separated from the raw milk by ultracentrifugation (UC), treatment with hydrochloric acid, or treatment with acetic acid, followed by filtration and preparation of the whey. The protein concentration of the whey was determined by spectrophotometry, and the size and concentration of EVs were measured by tunable resistive pulse sensing analysis. Surface marker proteins of EVs were detected by western blot (WB) analysis using the primary antibodies anti-CD9, anti-CD63, anti-CD81 and anti-MFG-E8.
Results: The UC method yielded a higher concentration of proteins in the whey than did acidification. However, both acidification treatments yielded higher amounts of EVs than UC. WB analysis revealed that acidification had partially degraded the surface marker proteins CD9 and CD81 but not CD63 or MFG-E8.
Summary/Conclusion: Acidification was likely favourable to the removal of casein and the rapid, efficient isolation of milk EVs. A higher amount of EVs were purified by acidification, but this treatment degraded partially some of the surface marker proteins of the EVs. Our results suggest that appropriate surface marker antigens should be used for evaluation of EVs from bovine milk after acidification in the following EVs experiments.
Funding: This study was partly supported by a research project for Improving Animal Disease Prevention Technologies to Combat Antimicrobial Resistance 2017–2021 FY of the Ministry of Agriculture, Forestry and Fisheries of Japan. This study was also supported in part by the OGAWA Science and Technology Foundation and the Morinaga Foundation for Health and Nutrition.

PF10.08

Comparison of isolating method for obtaining extracellular vesicles from cow's milk

Mai Morozumi[a], Hirohisa Izumi[b], Muneya Tsuda[c], Takashi Shimizu[a] and Yasuhiro Takeda[a]

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Introduction: MicroRNAs (miRNAs) are present in many foods including milk, which could be involved in various bioactivities when taken orally. Milk consists mainly of two fractions, i.e. casein and whey, and most of the milk miRNAs are thought to be included in extracellular vesicles (EVs) in whey fraction. Biological roles of milk miRNAs are not fully elucidated and thus require further investigation. However, procedures for isolating milk-derived EVs (M-EVs) have not fully established. The aim of this study was to compare methods for isolating M-EVs.
Methods: Aiming to minimize the contamination of casein in whey fraction, which is the great obstacle to determining M-EVs purity, whey fraction was separated from milk (defatted) by centrifugation only, acetic acid precipitation, or EDTA precipitation (n = 3). M-EVs were then isolated from each whey fraction by ultracentrifugation, an exoEasy Maxi kit

(Qiagen), a qEV kit (Izon Science) or an EVSecondL70 kit (GL Sciences). The number of M-EVs particles was measured using NanoSight (Malvern Instruments).
Results: Acetic acid precipitation prevented casein contamination to greater extents. Three combinations, such as "acetic acid precipitation and qEV", "acetic acid precipitation and EVSeocondL70" and "EDTA precipitation and qEV" were able to collect larger numbers of total M-EVs particles than the other combinations. Among the three combinations, "EDTA precipitation and qEV" achieved collecting the largest number of M-EVs but "acetic acid precipitation and EVSeocondL70" was able to obtain M-EVs fractions with high concentration.
Summary/Conclusion: The combination of "EDTA precipitation and qEV" is suited to collect the largest amount of M-EVs. The combination of "acetic acid precipitation and EVSeocondL70" is capable of obtaining M-EVs fractions with high concentration.

PF10.09

Generating, characterizing and testing recombinant extracellular vesicles as biological reference material

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Introduction: Recent years have seen a tremendous increase in the study of extracellular vesicles (EV) geared towards biological understanding, diagnostics and therapy. Concurrently EV data interpretation remains challenging owing to the complexity of biofluids and the technical variation introduced during EV sample preparation and analysis.
Methods: To understand and mitigate these limitations we have developed a standard operating procedure to generate trackable recombinant EV (rEV).
Results: Employing complementary characterization methods we demonstrate that rEV are stable, commutable and share both physical and biochemical characteristics with sample EV. rEV can be accurately measured using fluorescence-, RNA and protein-based technologies. Implementation of rEV reduces intra-method and inter-user variability of EV sample preparation and analysis, and improves the sensitivity of EV enumeration in biofluids.
Summary/Conclusion: The informed use of rEV will aid method development, instrument calibration, data normalization and routine evaluation of EV sample preparation and analysis in various research and biomedical applications.

PF10.10

ExtraSome: method for exosome isolation based on polyethylene glycol

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Introduction: Exosome sized 30-120 nm secreted from cells and present in blood, urine and cell media. It contains biomarkers that play important roles cell–cell communication. Therefore, it is important to isolate exosome in stable and effectively eliminate these contaminants. Extant method to isolate exosome include ultracentrifugation, immunoisolation and precipitation in polymeric solution. Ultracentrifugation is the most conventional method due to its reliability but it has the demerits of lengthy and laborious centrifugation, requirement for expensive equipment and low yield. Immunoisolation which uses beads conjugated with an antibody to isolate EVs; this method has high specificity but the EVs are hard to detach from beads, and detachment methods may reduce the functionality of the surface protein.
Methods: Exosomes were isolate from Fetal Bovine serum (FBS): After centrifugation at 2000*g* for 30 min, 5 mL of FBS were combined with PEG buffer solution, resulting in 2–30% final PEG concentration. The sample were carefully mixed and incubated at 4 C overnight. Then the samples were pun down at 11,000 rpm for 1 h. The supernatant was discarded, and the exosome pellet was resuspended in PBS and the number of exosomes was quantified on a Nanosight LM10 instrument.
Results: We isolate exosome from FBS using PEG buffer solution varied molecular weight (1000, 6000, 8000, 10,000, 20,000) at various concentration (2–30 w%). We confirm the size, morphology, chemical structure and biological marker (CD63, CD81) of the exosome. As a result, we verify the optimal isolation condition of exosome for efficient system.
Summary/Conclusion: In summary, we have developed a new method for the determination of the critical PEG value of exosome, which was proven to be very convenient and reliable. We believe that this method is highly effective and economical and has great potential to be further used for the selective separation of exosome.
Funding: This work was supported by a National Research Foundation (NRF) grant funded by the Korean government, Ministry of Education and Science Technology (NRF-2017M2A2A6A01071157, NRF-2018R1C1B6008799).

PF10.11=OWP3.05

Aqueous two-phase system to isolate extracellular vesicles for prostate cancer diagnosis

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Introduction: Analyzing extracellular vesicles (EVs) is an attractive means in prostate cancer diagnosis. However, existing methods of EVs isolation have low efficiency, purity and long process time, which induce low diagnostic ability. To approach the problems, we adapt a two-phase system to diagnose prostate cancer by isolating EVs from patients' urine. Using the two-phase system, prostate hyperplasia (BPH) patients and prostate cancer (PCA) patients were diagnosed, and the diagnostic ability was compared with conventional diagnostic methods.

Methods: Forty-two prostate cancer (PCA) patients and 20 benign prostate hyperplasia (BPH) patients' urine, plasma, saliva was collected and used for identifying EVs isolation ability of aqueous two-phase system (ATPS) and for comparing diagnostic ability of ATPS with conventional diagnosis.

Results: With an optimized ATPS, EVs were isolated with an efficiency of approximately 90%. In addition, the EV-isolation time was within approximately 30 min, and the purity of EVs in ATPS was approximately two times better than achieved with a conventional methods, ultracentrifugation and polymeric precipitation. After the ATPS isolated EVs from patients' body fluid, PCR and ELISA were utilized to detect EVs derived from prostate cancer cells. The expression levels of RNA and protein markers of prostate cancer were compared, and the relationship between expression levels and clinical data was analysed. The results demonstrated that diagnostic ability based on ATPS was better than other conventional methods (serum PSA and sediments). Moreover, sensitivity increased by at least 10%, and specificity was improved by at least 20% compared to conventional methods.

Summary/conclusion: High quality and quantity of EVs can be obtained from patients' body fluid using ATPS. Using the abundant sources, which contain cancer-related proteins and genes, we can perform a diagnosis with high specificity and sensitivity. Therefore, ATPS offers a powerful tool for more specific and sensitive diagnosis.

PF11: EV-Based Therapeutics II
Chairs: Yasnouri Fujita; Xue Zou
Location: Level 3, Hall A

15:30–16:30

PF11.01

Therapeutic effect of plant sap-derived nanovesicles on cancer cells

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Introduction: Most of the chemical agents are toxic to both malignant and normal cells. The new anticancer agents with debilitating side effects are highly demand. Various plant sap have known to possess therapeutic effects like anticancer traditionally. Plant-derived nanovesicles play critical roles in intercellular and inter-species communications to transfer plant components to mammalian cells. Plant sap-derived nanovesicles successfully delivered contained components into cells with high efficiency.

Methods: We extracted plant sap-derived nanovesicles from four endemic plants: *Dendropanax morbifera* (DM), *Pinus densiflora* (PD), *Chamaecyparis obtusa* (CO) and *Thuja occidentalis* (TO), and investigated endocytosis pathway of nanovesicles to malignant and benign cells. We assessed their anti-cancer effects on breast, skin, colon and melanoma cancer cells of normal, benign and malignant origins.

Results: We found that different endocytosis pathway between malignant and benign cells, DM-derived exosome-like nanovesicles (DM-ENVs) showed anticancer effect especially on malignant breast cancer cells, while no cytotoxic effects were exhibited against benign cells. PD-ENVs showed the cytotoxic effect on malignant skin cancer cells but not on Fibroblasts. TO-ENVs and CO-ENVs showed no cytotoxic effect on most malignant cancer cells. We also found the synergistic effect of the DMNVs and PDNVs on malignant breast and skin cancer cells. We identified that combination of DM-ENVs and PD-ENVs make enhancement in the cytotoxicity against malignant cells than normal and benign cells.

Summary/Conclusion: We confirm that DM-ENVs have anticancer effects against malignant breast and skin cancer cells than benign breast and skin cancer cells. We also found synergistic effects according to the combination of DM-ENVs and PD-ENVs on malignant cells. These results provide that plant sap-derived ENVs can be a new source for specific cancer therapeutics.

Funding: This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the ministry of Education, Science and Technology (NRF-2016R1C1B2013345) and Samsung Research Funding Center of Samsung Electronics under Project Number SRFC-IT1701-00

PF11.02

Amniotic fluid stem cell extracellular vesicles derived from different species contain evolutionarily conserved microRNAs: valuable resources for regenerative medicine.

Lina Antounians and Augusto Zani

The Hospital for Sick Children, Toronto, Canada

Introduction: Amniotic fluid stem cells (AFSCs) are a population of multipotent cells that have been reported to hold broad regenerative potential. This regenerative capacity has been linked to a paracrine mechanism mediated by microRNAs (miRNAs) contained in AFSC extracellular vesicles (EVs). Herein, we investigated the miRNA content of AFSC-EVs from multiple species to identify commonly shared and evolutionarily conserved miRNAs that may be responsible for AFSC beneficial effects.

Methods: In this study, we combined data from the literature and from our laboratory.

Literature review: Using a defined strategy, we conducted a systematic review searching for studies reporting on AFSC-EVs and we extracted available miRNA sequencing data.

Our study: Rat AFSCs were subjected to exosome-depleted FBS in minimal essential media for 18 h. Conditioned medium was collected, cleared of cells and debris, filtered through a 0.22 μm syringe filter, and ultracentrifuged for 14 h at 100,000*g*. EVs were assessed for size (nanoparticle tracking analysis), morphology (transmission electron microscopy) and expression of canonical protein markers CD63, Hsp70, Flo-1 and TSG101 (Western). AFSC-EV RNA was isolated using SeraMir, constructed into libraries (CleanTag Small RNA) and sequenced on NextSeq

High Output single-end sequencing run. TargetScan was used to identify species-specific and evolutionarily conserved miRNA using seed sequences across all three species. Pathway enrichment analysis was conducted using miR-path.
Results: Overall, data on AFSC-EVs from three species (n = 2 human, n = 2 mouse, n = 1 rat) were included. Four miRNAs (miR-21, miR-24, miR-100 and miR-145) were found in AFSC-EVs from all three species and were reported to exert beneficial effects on lung, muscle and kidney regeneration. These miRNAs were enriched in signalling pathways that involve TGF-β (p = 0.004) and TNF-α (p = 0.03) and the maintenance of stem cell pluripotency (p = 0.0001). We also observed species-specific miRNAs (n = 15 human, n = 6 mouse, n = 6 rat) contained in AFSC-EVs.
Summary/Conclusion: AFSC-EVs isolated from different species have some miRNAs that are shared and evolutionarily conserved. These miRNAs may have a specific role in the regenerative effects that AFSC-EVs exert in different diseases.
Funding: CIHR-SickKids Foundation

PF11.03

Extra-cellular vesicles in human platelet lysates for clinical use and human cell *in vitro* propagation

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Introduction: Human platelet lysates (HPLs) are increasingly used in regenerative medicine and cell therapy. The functional activity of HPLs to regenerate tissues *in vivo* and to expand cells *in vitro* is believed to be due to their richness in a plethora of growth factors. However, little is known about the presence and content of extracellular vesicles (EVs) in HPLs, as well as their potential functional activity, biological impact and involvement in tissue repair. We aim to characterize the number, the size and the biological functions of EVs present in HPLs in order to develop dedicated preparations best suitable for specific clinical applications
Methods: Clinical grade of platelet concentrates were processed into different fractions of HPLs: platelet pellet lysate (PPL); heat-treated PPL ; serum-converted platelet lysate (SCPL) using calcium chloride/glass bead treatment; and heat-treated SCPL (HSCPL). EVs in HPLs were isolated using size exclusion chromatography column. The number and the size distribution were determined by dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). EVs functional activity was assessed for the expression of tissue factor and phosphatidylserine (PS) activity. In addition, the HPLs were tested for their thrombin and plasmin activity, anti-oxidative property and thrombin generation capacity
Results: Abundant number of EVs (10^{10} ~ 10^{12}/mL) was found in all HPLs fractions. DLS analysis showed that isolated EVs in PPL, HPPL, SCPL and HSCPL have size distribution approximately ranging as follows: 100 ~ 250 nm; 45 ~ 210 nm; 145 ~ 230 nm and 55 ~ 180 nm, respectively, these data being confirmed by NTA and TEM. None of the HPLs were found to have detectable TF-expressing EVs but some significant differences in PS-expressing EVs, as well as thrombin, plasmin and anti-oxidative activity were found, possibly linked to their EVs composition
Summary/Conclusion: This study establishes that all HPLs evaluated have a high content of EVs. Differences in functional activity were also unveiled supporting the need for further studies of the physiological functions of HPL-derived EVs in cell-based and preclinical animal models

PF11.04

EV-mediated delivery of enzymatically fabricated size-controllable functional DNA/RNA microstructures for therapeutic applications

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Introduction: Nucleic acids have been widely investigated as a generic material with the advantage of their wide range of biological functions, predictable molecular structure, programmability and reversibility of base-paring. Here, the following enzymatically fabricated multiscale functional nucleic acid-based structures are introduced: bubbled RNA-based cargoes (BRCs), messenger RNA nanoparticles (mRNA-NPs) and CpG incorporated DNA microparticles (CpG DNA-MPs).
Methods: Primer DNA and long linear DNA bearing complementary sequences to functional DNA or RNA were prepared for the synthesis of circular DNA for the BRCs and CpG DNA-MPs. For mRNA-NPs, plasmid DNA bearing T7 promoter region, ribosome binding site and ORF was prepared. T7 RNA polymerase or Phi29 DNA polymerase were used to induce self-assembly of DNA or RNA-based structures, respectively.

Results: Adaptation and modification of rolling circle replication (RCR) approach has enabled generating these functional structures with desired sizes for demonstration of regulation of biological processes. The BRCs were designed to down-regulate the target gene upon introducing to cells by bearing specific cleavage sites for Dicer to generate functional siRNAs. Furthermore, manipulating template DNA to polymerase ratio in the modified RCR reaction enabled size-modulable synthesis of BRCs. We also adapted plasmid DNA as template DNA in RCR to fabricate mRNA-NPs for up-regulation of target gene expression. While the two studies were geared towards regulation of gene expression, we also demonstrated the fabrication of CpG DMA-MPs for boosting immune response by improving efficiency of antigen presentation in macrophages.
Summary/Conclusion: Since the pre-assembled structures have high cargo capacity without any condensation, loading extracellular vesicles (EVs) with the DNA/RNA-based nanostructures would greatly enhance therapeutic potential of nucleic acid-based therapeutics. Together with the nature of EVs being safe and effective delivery agent, we envision that the exploitation of EVs in the field of nucleic acid engineering would provide a way of next-generation gene therapy.
Funding: This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation of Korea (NRF) funded by the Korean Government (NRF-2016M3A9C6917402).

PF11.05

VES4US: Extracellular vesicles from a natural source for tailor-made nanomaterials

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Introduction: VES4US is a new European project funded by the Horizon 2020-Future and Emerging Technology Open programme, which aims to develop an innovative platform for the efficient production of extracellular vesicles (EVs) from a renewable biosource, enabling their exploitation as tailor-made products in the fields of nanomedicine, cosmetics and nutraceutics (https://ves4us.eu). A core aspect of the project is to focus on an identified natural source to constitute a cost-effective and sustainable source of EVs. The project will run for the next three years with six organizations from six European countries.
Methods: In the first phase, the focus will be the selection of the natural source and the optimization of culture condition at pre-industrial scale. Then, the isolation and physiochemical characterization of the EVs will be realized. In the second phase, the functionalization and load of the EVs will be approached. Finally, the biological activity of the EVs will be explored both *in vitro* and *in vivo*.
Results: The selection of the best EV-producing natural source strain/s is ongoing and it will get to the production of the EVs needed to develop the natural nanocarriers. Before that happens, it exists the need of applying good research practices that include personnel training on Standard Operating Procedures to control major experimental activities for harvesting, manipulating, storing, characterizing and treating EVs, as well as for key related activities.
Summary/Conclusion: Safe, efficient and specific nano-delivery systems are essential to current therapeutic medicine, cosmetic and nutraceutics sectors. The ability to optimize the bioavailability, stability and targeted cellular uptake of a bioactive molecule while mitigating toxicity, immunogenicity and off-target/side effects is of the utmost priority. VES4US aims at creating a fundamentally new bioprocessing approach to generate and functionalize EVs from a renewable biological source.
Funding: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 801338.

PF11.07

Therapeutic effects of mesenchymal stem cell exosomes in myocardial ischemia/reperfusion injury in a porcine model

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Introduction: After an acute myocardial infarction, restoration of the blood flow to the ischemic myocardium (reperfusion) is the standard and most effective treatment

for reducing the infarct size and improving clinical outcome. The process of reperfusion, however, can induce injury as well. Studies in animal models suggest that this "reperfusion injury" accounts for up to 50% of the final size of a myocardial infarct and in these models a number of strategies have been shown to ameliorate lethal reperfusion injury.

Our laboratory previously showed that human ESC-derived mesenchymal stem cell-derived exosomes mediate cardio protection during myocardial ischemia/reperfusion injury in a mouse model.

However, the therapeutic efficacy of these exosomes in a large-animal model remain to be addressed.

In the current study, we therefore hypothesized that infusion of MSC exosomes reduces infarct size and preserves cardiac function in a large-animal model.

Methods: Thirty female landrace pigs were subjected to 60 min transluminal balloon occlusion and treated with a combination of intravenous (1 mg) and intracoronary (1 mg) MSC exosomes (n = 15) or placebo (n = 15) in a randomized, blinded fashion. At baseline and prior to termination 3D transesophageal echocardiography was performed to assess cardiac function. Infarct size will be calculated as the percentage of the area at risk using Evans blue/TTC double staining.

Results: This is an ongoing study and no major results are available yet. However, pilot studies in an open chest LCx ligation model revealed that 1 mg of MSC exosomes (200 µg i.v. 5 min before reperfusion and 800 µg intracoronary immediately after reperfusion) resulted in a significant reduction of infarct size compared to saline injection (infarct size as a percentage of the area at risk 22% vs 46%, p = 0.01).

Summary/Conclusion: In this study, we will investigate whether MSC exosomes are capable of reducing infarct size and preserving cardiac function in a highly translational large-animal model.

The main goal is to establish a clinically applicable protocol for MSC exosomes to be used as a therapeutic tool for the treatment of acute MI.

Funding: Dutch Heart Foundation

PF11.08

The therapeutic potential of MSC-derived extracellular vesicles for stroke

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Introduction: Adult stem cell therapy is an excellent treatment for a variety of ischemic diseases including ischemic heart disease, limb ischemia and stroke. Mesenchymal stem cells (MSCs) exert their therapeutic capability via extracellular vesicles (EVs) in infarcted tissue. Therefore, we hypothesized that MSC-derived EVs (MSC-EVs) possess the equivalent sets of therapeutic molecules to MSCs.

Methods: EVs were isolated from 3D cultures of Wharton's Jelly MSC (WJ-MSC) using tangential flow filtration system and characterized by nanoparticle tracking analysis. We investigated the therapeutic efficacy of WJ-MSC derived EVs (does tested: 0.3 × 1010, 1.5 × 1010, 3 × 1010 EVs/rat) in a rat stroke model.

Results: The infarct size did not decrease, but the ladder walking test showed a greater improvement in behaviour in all EV groups than in the control group. Immunohistochemical analysis was performed using ki-67 (proliferating cells), DCX (immature progenitor neurons) and collagen IV (angiogenesis) of ipsilateral. EVs groups showed significantly increased co-expression of ki-67 and DCX in the subventricular zone. Enhanced expression of collagen IV in the ischemic boarder zone was more found in the EV groups than in the control group. Diffusion tensor imaging was used to examine the regeneration of nerve fibre bundles, and nerve fibre bundles were also increased in the EV groups.

Summary/Conclusion: Our study shows that WJ-MSC derived EVs from 3D culture system promotes the neurogenesis, angiogenesis and recovery of nerve fibre bundles in rat stroke models. These results suggest that an effective MSC-derived EV can be used ideally for the treatment of stroke.

Funding: This study was supported by a grant from the Korean Healthcare Technology R&D Project, Ministry of Health & Welfare (HI17C1256) and Basic Science Research Program, the Ministry of Science, ICT and Future Planning (2018M3A9H1023675).

PF11.10

Exosomes from human urine-derived stem cells promote neurogenesis via histone deacetylase6 regulation in ischemic stroke

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Introduction: Human urine-derived stem cells (USCs) are receiving much more attention in tissue regeneration and injury repair. Exosomes derived from USCs

(USCs-Exo) have recently been suggested to mediate the restorative effects of USCs. However, whether USCs-Exo play a protective role in stroke remains unknown.
Methods: Therapeutic effect of USCs-Exo *in vivo* was evaluated by cerebral infarction and neurological behaviour. The proliferation and differentiation of neural stem cells (NSCs) was determined by double immunofluorescent staining. The viability, apoptosis, proliferation, and differentiation of NSCs subjected to oxygen glucose deprivation/reoxygenation (OGD/R) were assessed, respectively. The protein expression and activity of HDAC6, and the expression levels of miRNAs both *in vivo* and *in vitro* were assessed to detect the possible mechanism.
Results: We found that intravenous injection of USCs-Exo reduced brain infarct volume and improved functional recovery by enhancing the proliferation and differentiation of NSCs in ischemic rats. The *in vitro* results suggested that USCs-Exo increase viability, reduce apoptosis, and promote the proliferation and neuronal differentiation of NSCs after OGD/R. We further found that miR-206 contained in USCs-Exo is associated with the therapeutic efficacy for neurogenesis through the inhibition of histone deacetylase6 (HDAC6).
Summary/Conclusion: Our study demonstrates that USCs-Exo can improve neurological function recovery by promoting neurogenesis, which is attributed to miR-206-mediated HDAC6 inhibition. Our research suggests that the use of USCs-Exo represents a novel therapeutic strategy for stroke recovery.
Funding: This work was funded by National Natural Science Foundation of China (Numbers 81671209, 81471243 and 81472152).

PF11.11

Protective effect of extracellular vesicles released from the neural stem cells on 6-hydroxydopamine induced pathological condition of Parkinson's disease

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Introduction: Parkinson's disease (PD) is a neurodegenerative disease characterized by bradykinesia, resting tremors and postural instability. A key symptom of PD is the loss of the nigral dopaminergic neurons and subsequent dopamine deficit in the brain. However, the precise mechanism is still unknown. While neural stem cells (NSCs) are potential therapeutic resources for PD, NSC-secreted extracellular vesicles (EVs) including exosomes are key mediators of positive paracrine effects. Direct evidence for neuronal protective effects of EVs is essential for developing new PD therapeutics.
Methods: To trace EV movement, a lentivirus containing Palm-tandem dimer tdTomato (Palm-td) was transduced into F3 NSCs. EVs isolated from Palm-td-infected F3 cells showed high tdTomato fluorescence intensity enough to visualize their functional actions such as secretion, migration and engulfment between cells. We found that pretreatment with EVs dramatically prevented 6-OHDA-induced toxicity by reducing intracellular reactive oxygen species (ROS), percentage of apoptotic cells and caspase-3/7 activity.
Results: These results indicate that NSC-derived EVs have neuroprotective effects against the cell damage, possibly through antioxidant and anti-apoptotic action. Specifically, F3-derived EVs effectively prevents 6-OHDA-induced the production of ROS, NSC-EVs that inhibit ROS production might be applied as an important therapeutic agent for neuroprotection. EV-mediated neuroprotection likely acts by inhibiting the generation of caspase-3/7, leading to reduce apoptosis induction caused by 6-OHDA neurotoxicity.
Summary/Conclusion: In summary, this study demonstrates that NSC-derived EVs protected dopaminergic cells from oxidative insults. While 6-OHDA-induced cell death in SH-SY5Y cells occurs by the generation of ROS, the neurotoxin-mediated cell death was suppressed by F3-derived EVs via their protective effects on ROS-induced cell damage. We therefore expect that further investigations into the therapeutic applications of NSC-derived EVs will reveal additional advantages for EV-based PD therapies in comparison to cell transplantation.

PF11.12

The function of extracellular vesicles secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells on a cellular ischemic stroke model

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Introduction: Stroke is the second leading cause of death and the primary cause of long-term disability, but yet lack of effective treatment. Studies suggested the transplantation of mesenchymal stem cells (MSCs) improved recovery from stroke in animal

models, similar therapeutic effect was found with the injection of MSCs medium. MSCs medium was found enriched with extracellular vesicles, hence leads to the focus on utilizing extracellular vesicles to treat neurological diseases, due to the evidence that extracellular vesicles are able to penetrate the blood–brain barrier. This project aims to develop a product with enriched extracellular vesicles and to evaluate its therapeutic efficacy in ischemic stroke.

Methods: MSCs, with same passage number, were derived from human-induced pluripotent stem cells-MSCs for the isolation of extracellular vesicles. The derived MSCs were then confirmed by the adherence to plastic, multipotent differentiation potential and surface antigen expressions. Three methods (ultracentrifugation, ultrafiltration and polyethylene glycol) were used to extract extracellular vesicles, which were further analysed by the expression of surface proteins, electron microscopy, ribosomal RNA detection and oxygen–glucose deprivation (OGD) *in vitro* stroke model.

Results: Differentiated MSCs exhibited adherence to plastic, ability to differentiate into osteoblasts, adipocytes and chondroblasts, and ≥95% population expressed CD105, CD73 and CD90, and lack of CD45, CD34 and HLA class II. The isolated extracellular vesicles expressed CD9, CD63 and CD81, with the size between 30 and 200 nm and contained RNA with a peak between 25 and 200 nucleotides. Products from ultrafiltration were found to increase cell viability *in vitro* stroke model most significantly.

Summary/Conclusion: Extracellular vesicles were able to increase the viability of neuronal cell (HT22) in oxygen–glucose deprivation *in vitro* stroke model, indicating the potential use of extracellular vesicles injection as an alternative therapy for ischemic stroke.

Funding: Innovation and Technology Fund ITS-053–17FX, the Government of the Hong Kong Special Administrative Region.

PF11.13

Endosomal escape enhancing compounds facilitate functional delivery of EV cargo

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Introduction: Extracellular vesicles (EVs) are desirable carriers and delivery vehicles for therapeutic cargoes. We aimed to load EVs with Cre recombinase (Cre) as a model protein cargo and determine whether functional delivery to cells could be improved by using uptake-enhancing compounds.

Methods: Expi293F cell line was used for isolating Cre loaded EVs by differential centrifugation after transfecting releasing cells with constructs for protein expression. EVs were then analysed by nanoparticle tracking analysis, western blotting, RT-qPCR and cryo-electron microscopy including detergent and nuclease digestion controls. Uptake of Cre loaded EVs was assessed using modified Hek293T cells expressing a fluorescent reporter cassette consisting of LoxP – GFP – LoxP – RFP.

Results: Endosomal escape enhancers chloroquine and Unc10217939 increased TATcre functional delivery by ≥50%. CreFRB protein was loaded into EVs by rapalog-induced dimerisation to CD81FKBP. Cells treated with 20 μg/mL CreFRB loaded EVs showed functional Cre activity only in the presence of 25 μM chloroquine or 2 μM unc10217939.

Summary/Conclusion: Passively loaded protein and mRNA was effectively delivered to recipient Hek293T fluorescent Cre reporter cells in the presence of endosomal escape enhancing compounds. This finding shows that endosomal escape enhancing compounds may have a place in the clinic to improve delivery efficiency of nanoparticle-based therapies.

PF11.14=OWP1.02

MSC exosome works through a multifaceted mechanism of action in joint repair

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Introduction: Mesenchymal stem cell (MSC) exosome is increasingly accepted as the principal agent that underpins the therapeutic efficacy of MSC in tissue repair. Here, we aim to elucidate the mechanism of action of MSC exosome in immunocompetent rat models of osteochondral defect and osteoarthritis (OA).

Methods: Exosomes were purified from conditioned medium of human MSCs by size fractionation. Osteochondral defect creation or anterior cruciate ligament transection to induce OA were performed in 72

adult rats. Thereafter, weekly 100 μl intra-articular injections of 100 μg exosome or PBS vehicle were given. Analysis included weight distribution, histology, immunohistochemistry and cytokine assay. Cellular assays using chondrocytes were performed to determine the exosome-activated cellular processes and signalling pathways.

Results: We observed that exosome-mediated repair of osteochondral defects was characterized by increased cellular infiltration and proliferation, enhanced matrix synthesis, together with a regenerative M2 macrophage phenotype and a reduction in pro-inflammatory cytokines IL-1β and TNF-α. In OA joints, MSC exosome mediated an early suppression of pain and degeneration with reduced inflammation, followed by sustained proliferation and matrix restoration that led to cartilage and subchondral bone regeneration. Using chondrocyte cultures, we could attribute some of these cellular activities during exosome-mediated joint repair to exosomal CD73-mediated adenosine activation of AKT and ERK signalling. These effects were partially abrogated by wortmannin or U0126, which inhibited AKT and ERK phosphorylation, respectively. The role of exosomal CD73 was confirmed using CD73 inhibitor and theophylline that showed inhibition of exosome-induced AKT and ERK phosphorylation.

Summary/conclusion: Our observations suggest that MSC exosome works through a multifaceted MoA that involved multiple cellular processes to restore joint homeostasis and promote regeneration.

Funding: National Medical Research Council Singapore (NMRC/CNIG/1168/2017 and NMRC/CIRG/1480/2017).

PF11.15=OWP1.04

Exosome-mediated enhancement of cellular therapy in acute myelogenous leukemia (AML)

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Introduction: Of the acute myelogenous leukemia (AML) patients able to tolerate curative therapy with chemotherapy and stem cell transplant, many are challenged by treatment related toxicities as well as graft versus host disease. There is novel work exploring the utility of haploidentical cellular therapy infusion in order to incite purposeful recipient immune response and subsequent cytokine storm to treat refractory AML. Our group has demonstrated the healing potential of bone marrow-derived mesenchymal stem cell extracellular vesicles (MSC-EVs) across multiple disease states, most recently demonstrating the pro-apoptoic signalling imparted by these nanoparticles on nascent leukemic cells *in vivo*; as well as the potentiating effects of MSC-EVs when used as an adjunct to standard cytarabine chemotherapy. We have also shown the protective role of HMSC EV on radiated BM and stem cell recovery.

Methods: Kasumi AML cells lines were seeded with MSC-derived EVs. Vesicles were isolated using an established differential centrifugation technique, and were co-cultured with Kasumi cells for various time points. To study cellular viability, we used a fluorescence-based method for quantifying viable cells.

We also explored various modes of death EVs may illicit via a tri-dye Abcam assay designed to simultaneously monitor apoptotic, necrotic and healthy cells. Both assays were used to measure viability and apoptosis in similar experiments employing cytarabine

Results: AML cell proliferation decreased after 1–6 days of co-culture with hMSC-derived EVs.

Apoptosis is the primary mode of death induced.

AML cell Proliferation Decreased synergistic after 1–6 days of co-culture with hMSC-derived EVs ± Cytarabine.

Summary/conclusion: MSCs inhibits the proliferation of the AML cell line *in vitro* and work synergistically with cytarabine chemotherapy to promote apoptotic death in AML cell lines. Our prior work has shown that MSC-EVs can abate the effects of toxic chemo/radiation and serve to protect stem cell allowing for quicker recover in cell blood counts.

Based on the innate ability of MSC-EV to directly alter the cellular machinery of abnormal leukemic cell and of nascent immune cells our corollary hypothesis is that BM-derived MSC-EVs may serve as suitable alternative to conditioning chemo/radiation in the AML setting and will enhance the effects seen by cellular therapy infusion.

Funding: t32

PF12: Advances in EV Cargo Profiling
Chairs: Leonid Margolis; Yutaka Naito
Location: Level 3, Hall A

15:30–16:30

PF12.01

Tumor driver TGFBR2-dependent microRNA profiles in colorectal cancer cells and their EVs

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Introduction: Microsatellite unstable (MSI) colorectal cancers accumulate frameshift mutations at short repetitive DNA sequences (microsatellites). MSI-specific mutation patterns in tumour driver genes such as *Transforming Beta Receptor Type 2* (*TGFBR2*) were found to be reflected in the cargo of MSI cell line-derived extracellular vesicles (EVs). In previous work, we have shown that TGFBR2 reprograms the protein content of MSI tumour cells and small EVs derived thereof. Here, we report on TGFBR2-dependent alterations of miRNA expression in small EVs and their corresponding parental MSI tumour cells.
Methods: To identify TGFBR2-regulated miRNAs in an isogenic background, the established doxycycline (dox)-inducible MSI model HCT116-TGFBR2 was used. RNA was isolated from four biological replicates of TGFBR2-proficient (+dox) and TGFBR2-deficient (-dox) cells and their EVs. EVs were isolated by differential centrifugation, ultrafiltration, and precipitation and characterized by electron microscopy, Western blot, and nanoparticle tracking. RNA quality and concentration were determined by capillary electrophoresis. cDNA libraries for small RNA fractions were generated and RNA sequencing was performed. TGFBR2-regulated miRNA expression was assessed by DESeq2 and validated by RT-qPCR.
Results: From 471 identified miRNAs, the majority (n = 263) was unaffected by TGFBR2 expression and shared by small EVs and parental MSI cells. In addition, we detected specific miRNAs exclusively present in EVs from TGFBR2-deficient (n = 4) or TGFBR2-proficient (n = 14) MSI cells. Differential expression analysis revealed TGFBR2-regulated miRNAs in EVs (n = 10) and MSI donor cells (n = 15). Three candidates (miR-381-3p, −889-3p, −323a-3p) were found to be upregulated in both TGFBR2-proficient EVs and parental cells.
Summary/Conclusion: Our results emphasize a broad overlap of miRNAs between EVs and their parental cells but also highlight the impact of the recurrent MSI tumour driver TGFBR2 on aberrant miRNA signatures in MSI cancer cells and their small EVs.
Funding: This work was supported by intramural funding from the Technical University Munich (MP) and the University Hospital Heidelberg (JG, JK).

PF12.02

Orthologous grouping and comparison of prokaryotic and eukaryotic EV proteomes

Tae-Young Roh[a], Seokjin Ham[b], Dae-Kyum Kim[c], Jaewook Lee[c] and Yong Song Gho[d]

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Introduction: Most prokaryotic and eukaryotic cells secrete extracellular vesicles (EVs) with bioactive molecules, including proteins and nucleic acid. Protein cargos important for EV biogenesis and/or biological functions can be found using proteomic analyses.
Methods: To analyse the similarity and difference between prokaryotic and eukaryotic EVs, EV protein databases was obtained from EVPedia (http://evpedia.info), regardless of EV sources and analysing platforms. EV proteins were catalogued into orthologous groups and annotated these groups using eggNOG database. Gene set enrichment analysis (GSEA) was employed to determine how much the orthologous groups are enriched in EVs of prokaryotic or eukaryotic species. The core network of prokaryotic and eukaryotic EV orthologous groups were explored by Generalized HotNet analysis. Only hot clusters with more than four orthologous groups were visualized by Cytoscape.
Results: A total of 6634 proteomic orthologous groups were identified from 33 prokaryotes and 22 and

separated into two distinct groups. Each orthologous group was annotated with gene symbols, GO terms, as well as functional interactions. Frequently detected orthologous groups were related with mainly membrane-associated compartments. The GSEA analysis showed some common and specific proteins to prokaryote or eukaryote in the categories of biological process and cellular component. The correlation network analysis clearly provided a domain-specific terms such as intracellular organelle cilium, cytoplasm ribosome, and ribosome proteasome complex for eukaryotes, and cytoplasm envelope, extracellular exosome and cell outer membrane for prokayrotes.

Summary/Conclusion: Our comprehensive EV proteome analysis could provide a functional modules related with characteristic biological mechanisms in prokayrotes and eukaryotes. This analytical strategy will also provide a new integrative method to investigate EV proteins and propose an evolutionary protein repertoire of EV.

PF12.03

Quantitative proteomic analysis of trypsin-treated extracellular vesicles to evaluate the real-vesicular proteins

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Introduction: Extracellular vesicles (EVs) are nano-sized vesicles surrounded by a lipid bilayer and released into the extracellular milieu by most of cells. Up to date, various isolation methods of EVs have been established. However, most of the current methods isolate EVs with the contaminated extravesicular proteins, which are co-isolated proteins or non-specifically bound proteins. Since it is difficult to isolate EVs without any contaminations, the evaluation of the real-vesicular proteins could be valuable for the quality control of EVs.

Methods: SW480 EVs were isolated from the conditioned medium by sucrose cushion and iodixanol buoyant density gradient ultracentrifugation. The isolated EVs were treated with trypsin or control for 6 h and then pelleted by ultracentrifugation, before undergoing LC-MS/MS.

Results: Trypsin treatment could digest the contaminated extravesicular proteins without influencing the intravesicular (luminal) proteins, as well as size and morphology of EVs. By the quantitative proteomic analyses between vesicular proteins with and without trypsin treatment, we classified the vesicular proteins into 363 candidate real-vesicular proteins and 151 contaminated extravesicular proteins. Protein interaction network analyses showed that candidate real-vesicular proteome is composed of proteins derived from plasma membrane (46.8%), cytosol (36.6%), cytoskeleton (8.0%) and extracellular region (2.5%). On the other hand, most of the identified proteins derived from other cellular organelles including nucleus, Golgi apparatus, endoplasmic reticulum and mitochondria were considered as the contaminated extravesicular proteins. In addition, protein complexes, including ribosome and T-complex proteins, were classified as the contaminated extravesicular proteins.

Summary/Conclusion: Taken together, this trypsin treatment to EVs with large-scale quantitative proteomics allows the evaluation of the real-vesicular proteins in isolated EVs as well as the sub-vesicular localization of identified proteins. Therefore, our results provide the applicable approach to identify the reliable diagnostic markers of EVs.

PF12.04

Characterization of sweat extracellular vesicles

Genevieve Bart[a], Anatoliy Samoylenko[a], Daniel Fischer[b], Anna Kaisanlahti[c], Artem Zhyvolozhnyi[a], Marko Suokas[d], Prateek Singh[a], Justus Reunanen[c] and Seppo Vainio[d]

[a]University of Oulu, Biocenter Oulu, Laboratory of developmental Biology, Oulu, Finland; [b]Natural Resources Institute Finland (Luke), Animal Genomics, Jokioinen, Finland; [c]University of Oulu, Biocenter Oulu, Cancer and Translational Medicine Research Unit, Oulu, Finland; [d]University of Oulu, Biocenter Oulu, Department of Biology, Oulu, Finland; [e]University of Oulu, Biocenter Oulu, Laboratory of Developmental Biology, Oulu, Finland

Introduction: The view that human beings are more complex than originally thought and could be described as a mixture of human and microorganism is gaining momentum and even biofluids which had always been considered sterile have now been shown to contain bacteria originating molecules and, in some cases, bacteria. Healthy human skin is populated by many species of unicellular organisms, a number of which are known to secrete extracellular vesicles (EVs). Our study of sweat EV cargo using omics is aiming to shed some light on these complex interactions.

Methods: We have collected sweat from the upper body of exercising individuals (men and women) and isolated EVs and EV RNA using concentration and filtration. EVs were checked by TEM and NTA then subjected to proteomics analysis. For RNA extraction EVs were directly lyzed on filter. 1–10 ng of RNA was used to make libraries for sequencing. Filtered and trimmed reads were aligned to human genome using Bowtie.

Unmapped reads were blasted against the EMBL database to identify and classify metagenomics reads.
Results: A few hundred human proteins were identified but also a number of bacterial proteins. In the case of RNA the number of unmapped reads was larger than is usually observed with extracellular small RNA sequencing. Metagenomic analysis provided information about species but only a certain number of reads could be assigned, probably due to the lack of available genome data. There is also an uncertainty about the precise species as we can only identify with any precision taxonomy at the level of order.
Summary/Conclusion: Sweat EVs are a mixture of human and microbe-derived EVs and their complete characterization will depend on the availability of genomic information including for difficult to cultivate strains.
Funding: Academy of Finland Biofuture2025

PF12.05

Proteomic signature of mesenchymal stromal cell-derived small extracellular vesicles.

Bas WM. van Balkom[a], Hendrik Gremmels[a], Bernd Giebel[b] and Sai Kiang Lim[c]

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Introduction: Small extracellular vesicles (EVs) are 50–200 nm vesicles secreted by most cells. They are considered as mediators of intercellular communication, and EVs from specific cell types, in particular mesenchymal stem/stromal cells (MSCs), offer powerful therapeutic potential, and could provide a novel therapeutic strategy. They appear promising and safe (as EVs are non-self-replicating), and eventually MSC-derived EVs (MSC-EVs) may be developed to standardized, off-the-shelf allogeneic regenerative and immunomodulatory therapeutics. Promising preclinical data have been achieved using MSCs from different sources as EV-producing cells. Similarly, a variety EV isolation and characterization methods have been applied. Interestingly, MSC-EVs obtained from different sources and prepared with different methods show *in vitro* and *in vivo* therapeutic effects, indicating that isolated EVs share a common potential.
Methods: We analysed published MSC-EV proteomics datasets to identify a unique and robust proteomics signature.
Results: Here, we compare well-characterized and controlled publicly available proteome profiles of MSC-EVs to identify a common MSC-EV protein signature that might be coupled to the MSC-EVs' common therapeutic potential.
Summary/Conclusion: This protein signature may be helpful in developing MSC-EV quality control platforms required to confirm the identity and test for the purity of potential therapeutic MSC-EVs.

PF12.06

Comparative analysis of stool extracellular vesicles between germ-free, bifidobacteria-di-associated and SPF mice

Hirohisa Izumi[a], Tatsuya Ehara[b], Mai Morozumi[b], Fuuka Tabata[b], Yosuke Komatsu[b], Takashi Shimizu[b] and Yasuhiro Takeda[b]

[a]Morinaga Milk Industry Co., Ltd., Zama-city, Japan; [b]Morinaga Milk Industry Co., Ltd., Zama-City, Japan

Introduction: Gut microbiota is closely related to host immune and metabolic functions. Recently, microRNA (miRNA) in extracellular vesicles (EVs) produced by host intestinal epithelial cells participate in shaping the gut microbiota. However, effects of microbiota on host gut EVs and miRNAs are not fully elucidated. In this study, we investigated the effects of microbiota on host stool EVs and miRNAs using germ-free (GF), bifidobacteria-di-associated, and specific pathogen free (SPF) mice.
Methods: GF mice and SPF mice (11 week, male) were obtained. At 12-week-old age, part of GF mice were inoculated with *Bifidobacterium longum* BB536 and B. breve M-16V (1109 each) once, and maintained with 5% fructooligosaccharides (bifidobacteria-di-associated mice). Mice were sacrificed at 15-week-old age by deep anaesthesia with sevoflurane, and tissue samples and stool samples were harvested. Stool EVs were purified using exoEasy Maxi Kit, and were analysed by NanoSight LM10. Total RNAs were purified from stool EVs using miRNeasy, and were analysed by microarray.
Results: NanoSight analyses revealed that mean EV size of GF stool was significantly smaller than that of SPF stool, and EV quanity of bifidobacteria-di-associated stool was tend to be increased than that of GF stool. Moreover, microarray analysis revealed that microbiota could change miRNA expression profiles of stool EVs.
Summary/Conclusion: Microbiota might affect host gut function via alterations of EVs' characteristics.

PF12.07=OWP3.07

Shed microvesicles released from human primary and metastatic colorectal cancer cell lines contain key cancer progression proteins and RNA species

Wittaya Suwakulsiri[a], Alin Rai[a], Rong Xu[a], Maoshan Chen[a], David Greening[b] and Richard Simpson[a]

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Introduction: Extracellular vesicles (EVs) function in bidirectional cell–cell communication and contribute to the sustained growth, invasion, and metastasis of cancer cells within the tumour microenvironment (TME). EVs comprise two main classes – exosomes and shed microvesicles (sMVs, also termed microparticles and ectosomes) – with distinct modes of biogenesis. Within each EV class, subtypes exist that can be distinguished by their distinct protein/RNA signatures. Whilst much is known about exosome cargo content and functionality, sMVs are poorly understood.
Methods: Here, we compare protein/RNA profiles and functionality of sMVs and exosomes secreted from human primary (SW480) and metastatic (SW620) colorectal cancer cell lines. Milligram amounts of EVs were purified from cell culture media using a combination of differential ultracentrifugation/isopycnic iodixanol density centrifugation. Label-free quantitative mass spectrometry was performed to obtain protein profiles for SW480-derived and SW620-derived sMVs.
Results: We show that sMVs, unlike exosomes, are ALIX-, TSG101-, CD63- and CD9- and contain a different suite of key cancer progression modulators. Protein/RNA signatures for SW480-derived sMVs and exosomes differ from each other and also from their SW620-derived counterparts. SW480-derived sMVs are enriched in ITGA/B, ANXA1, CLDN7, CD44 and EGFR/NOTCH signalling networks, while SW620-derived sMVs are enriched in PRKCA, MACC1, FGFR4 and MTOR/MARCKS signalling networks. Fibroblast invasion capabilities of SW480-derived and SW620-derived sMVs are comparable.
Summary/conclusion: Furthermore, we report for the first time a comprehensive biochemical/functional analysis of a hitherto undescribed subpopulation of sMVs. We anticipate our *in-vitro* findings will be a starting point for more sophisticated studies aimed at elucidating the biochemical and functional properties of EV subtypes *in vivo*. The emerging roles of specific EV subtypes in the TME we believe will alter our view of cancer biology and might present new targets for therapeutic intervention.
Funding: Funding support from La Trobe University, Melbourne, Australia.

PF12.08=OWP3.08

Mass spectrometry analysis of small extracellular vesicles isolated from ovarian cancer ascites

Anna Kotrbová[a], Kristina Gomoryova[a], David Potesil[b], Vit Weinberger[c], Igor Crha[c], Eva Jandakova[d], Lubos Minar[c], Zbynek Zdrahal[b], Vítězslav Bryja[a] and Vendula Pospíchalova[a]

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Introduction: High-grade serous carcinoma (HGSC) of the ovaries, fallopian tube and peritoneum is the deadliest gynaecological malignancy with 5-year survival rate below 30%. HGSC is frequently accompanied by ascites, a pathological accumulation of fluid in the peritoneum, which can be exploited as a liquid biopsy containing not only cancer cells but also the tumour microenvironment including extracellular vesicles (EVs). Tumour cells produce substantially more EVs than healthy cells, thus malignant ascites is the source of enriched pool of EVs of HGSC origin.
Methods: Ascitic fluids depleted of cells were fractioned using size-exclusion chromatography and two fractions – containing and not containing EVs – were further analysed. In parallel, small EVs were also isolated from ascitic fluids using differential ultracentrifugation followed by purification step in sucrose/D2O cushion. In total, 24 malignant ascites and 5 non-malignant ascites were used for EV isolation and further analysed using high resolution hybrid mass spectrometer Orbitrap Fusion Lumos Tribrid. The subsequent data visualization and statistical analyses were performed using in-house developed pipelines in KNIME environment.
Results: We identified 2441 proteins in total in the EVs from the ascites among which 21 were present in all 29 EV samples and not in non-vesicular fractions. Several of these proteins were specifically enriched in small EVs in malignant ascites in comparison with non-malignant ascites. These proteins are now being evaluated as biomarkers.
Summary/conclusion: Using advanced mass spectrometry, we identified candidate proteins which are specifically enriched in small EVs of HGSC. These proteins warrant further investigation as they may act as important players in HGSC progression as well as

serve as potential prognostic/diagnostic/screening biomarkers of HGSC.

Funding: Czech Science Foundation, Grant No. GJ17-11776Y

PF12.09=OWP1.05

Extracellular vesicles derived from amniotic fluid stem cells rescue impaired foetal lung development via the release of microRNAs

Lina Antounians, Vincenzo Catania, Benjamin Liu, Areti Tzanetakis, Louise Montalva and Augusto Zani

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Introduction: Incomplete lung development, also known as pulmonary hypoplasia (PH), is a recognized cause of neonatal death. To date, there is no effective treatment that promotes foetal lung growth and maturation. Herein, we describe a stem cell based approach that enhances foetal lung development via the administration of extracellular vesicles (EVs) derived from amniotic fluid stem cells (AFSCs) in rat models of PH. Moreover, we report the microRNAs present in AFSC-EVs that are responsible for these beneficial effects.

Methods: AFSC-EVs were isolated by ultracentrifugation from conditioned medium (CM) of c-Kit+ rat AFSC that were grown in exosome-depleted FBS for 18 h. AFSC-EVs were assessed for size (nanoparticle tracking analysis), morphology (TEM), and expression of CD63, Hsp70, Flo-1 and TSG101 (Western).

Ex vivo: Pregnant dams were gavaged nitrogen at E9.5 to induce foetal PH. At E14.5, foetal lungs were harvested, and incubated with culture medium alone, AFSC-CM, or AFSC-EVs. Foetal lungs from untreated dams served as control. Lungs were compared for terminal bud density and surface area at 72 h, by two independent investigators.

In vitro: Foetal rat lung organoids were generated with epithelial cells from normal and hypoplastic lungs. Organoids were cultured for 10 days in either medium alone or medium supplemented with AFSC-EVs. Lung organoids from untreated normal pups served as control. Organoids were assessed for proliferation (Ki67) and markers of epithelial cell differentiation via immunofluorescence.

RNA-sequencing: RNA was isolated using SeraMir, constructed into libraries (CleanTag Small RNA), and sequenced on NextSeq High Output single-end sequencing run.

Results: Administration of AFSC-EVs increased terminal bud density and surface area of lung explants back to control levels and promoted lung epithelial cell differentiation in lung organoids (increased SPC and CC10 expression). AFSC-EVs contain 901 microRNAs, some of which are crucial for foetal lung development, such as miR17 ~ 92 cluster.

Summary/conclusion: Administration of AFSC-EVs rescues impaired foetal lung development in experimental models of PH. AFSC-EV regenerative ability is exerted via the release of miRNAs some of which regulate genes involved in foetal lung development. AFSC-EVs represent a promising therapeutic strategy for PH in foetuses.

Funding: CIHR-SickKids Foundation

PF12.10=OWP2.10

HIV-specific antibody-mediated targeting of ENV+ tissues by exosomes

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Introduction: ART (Antiretroviral Therapy) can effectively suppress HIV replication in the peripheral blood to an undetectable level. However, efforts to eradicate the latent virus in reservoirs remain a challenge and are a major obstacle in the treatment of HIV patients. Exosomes exhibit huge promise as an endogenous drug delivery nanosystem for delivering drugs to reservoir tissues given their unique properties, including low immunogenicity, innate stability, high delivery efficiency and mostly importantly the ability to penetrate solid tissues due to their lipophilic properties.

Methods: In this study, we engineered and expressed the ScFv of a high affinity HIV-specific monoclonal antibody, 10E8, on exosome surface. Exosomes from 293T cells were loaded with curcumin via saponin, with efficient up to 34%. 10E8ScFv-expressing exosomes (10E8-Exo) showed highly efficient targeting of and curcumin delivery to CHO cell that expresses a trimeric gp140 on its surface (ENV+ cells) *in vitro* as demonstrated by confocal imaging and flow cytometry. We showed that 10E8-Exo could effectively bind to CHO cell that expresses a trimeric gp140 on its surface. The exosomes loaded with curcumin, a chemical that was shown to kill HIV-infected cells, showed specific killing of the trimeric gp140-expressing CHO cells. In an NCG mouse model that was grafted with the tumourigenic gp140-CHO cells and developed solid tissue tumours intravenously injected 10E8-Exo targeted the ENV-expressing tissues and delivered curcumin to induce a strong suppression of the ENV+ tumour growth with a low toxicity.

Results: Our results demonstrated that engineered exosomes can deliver anti-HIV agents to solid tissues by

specifically targeting cells expressing viral ENV and induce cell killings.

Summary/conclusion: It suggesting that such an approach can be developed for eradicating virus-infected cells in tissue reservoir

Funding: This study was supported by The National Key Research and Development Program of China (2016YFC1201000), Nature Science Foundation of Jiangsu Province (BY2015069-02) and National Nature Science Foundation of China (81672020). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Late breaking- EVs and cancer
Chairs: Sonia Melo; Golnaz Morad
Location: Level 3, Hall A

15:30–16:30

LBF01.01

Exosomes from LNCaP cells promote the activity of osteoblasts through the transfer of mir-375

Yun Ye[a] and Su-liang Li[b]

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Introduction: Studies have shown that exosomes influence tumour metastasis, diagnosis and treatment. It has been found that exosomal miRNAs are closely linked to the metastatic microenvironment. However, the regulatory role of exosomes from prostate cancer (PCa) cells in bone metastasis remains poorly understood.
Methods: We isolated and purified exosomes by ultracentrifugation, isolated total RNA from cells and total miRNA from exosomes, and analysed the level of miR-375 by RT-PCR. Exosome libraries from LNCaP cells and RWPE-1 cells were sequenced and filtered with an Illumina HiSeqTM 2500 system. The activity of alkaline phosphatase, the extent of extracellular matrix mineralization and the expression of osteoblast activity-related marker genes were measured to evaluate osteoblast activity.
Results: Morphological observation, particle size analysis and molecular phenotyping confirmed that the isolated extracts contained exosomes. Differential expression analysis confirmed the high expression of miR-375 in LNCaP cell-derived exosomes. We further determined which exosomes could enter osteoblasts and increase their miR-375 level. In addition, exosomal miR-375 could significantly promote the activity of osteoblasts.
Summary/conclusion: This study lays the foundation for further investigations on the function of exosomal miR-375 in the activation and differentiation of osteoblasts and the mechanism of bone metastasis in PCa.
Funding: none

LBF01.02=OWP1.13

Colorectal cancer cell-derived exosome enhances microenvironmental angiogenesis through modulation of intracellular metabolism

Atsushi Ikeda[a], Satoshi Nagayama[b] and Koji Ueda[c]

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Introduction: For improvement of prognosis of colorectal cancer (CRC), detection at an earlier stage of CRC is essential. Exosomes are nanovesicles secreted from plasma membrane, and have potential to be served as biomarker carriers. In this study, we performed proteomic profiling of exosomes secreted from viable CRC tissues.
Methods: To identify early detection biomarkers for CRC, we performed comprehensive proteome analysis of tissue-exudative extracellular vesicles (Te-EVs), which were obtained from culture media of freshly resected viable CRC tissue or adjacent normal mucosa (n = 17). Among the identified Te-EV proteins, we narrowed down the biomarker candidate by selecting proteins which are statistically upregulated ($p < .05$, fold change > 5.0) in Te-EVs from CRC tissues than those from adjacent normal tissues. Then we performed functional analysis of the biomarker candidate specifically.
Results: Comprehensive LC/MS analysis identified 6149 Te-EV proteins, in which 641 proteins showed significant upregulation in Te-EVs from CRC tissues ($p < .05$, fold change > 5. 0) compared to those from adjacent normal mucosa. We focused especially on GAM ($p = 7.0 \times 10^{-5}$, fold change = 7.4) as a novel biomarker candidate. GAM protein was significantly overexpressed in CRC tissues compared with adjacent normal mucosa. In EV-sandwich ELISA assay, the expression level of GAM on plasma EVs from CRC patients was significantly higher than that from healthy donors in EV-sandwich ELISA assay ($n = 133$, $p = 4.0 \times 10^{-7}$). In addition, the uptake of GAM-overexpressing EVs enhanced vascular endothelial cell growth and angiogenesis through modulation of nitric oxide metabolism.
Summary/conclusion: EV-GAM might have great potential as a target for both CRC diagnosis and therapy. Our strategy for identification of exosomal biomarker by proteomic profiling of Te-EV proteins can be applied to other cancers.

LBF01.03

Augmentation of Exosome secretion in Ha-Ras^{V12}-induced cell softening and multilayer cellular aggregates in Madin-Darby canine kidney cells

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Introduction: Ha-Ras^{V12} overexpression-induced cell softening and multilayer cellular aggregates (cell clumping) during confluence in MK4 cells (inducible Ras harbouring MDCK cells). We have demonstrated that Ha-Ras^{V12}-induced cell softening and transformation required Cav-1 downregulation and augmented secretion of Wnt-5a containing exosome. Here, we explored the role of exosome secretion and cell softening in Ha-Ras^{V12}-induced cell clumping.
Methods: We employed MK4 cells (inducible Ha-Ras-harbouring MDCK cells). Cell stiffness was measured by atomic force microscope (Biowizard II, JPK) and cell aggregates were examined under confocal microscope. Quantitation of exosome secretion in culture media was assessed by Nanoparticle analysis.
Results: We found that Induction of Ha-Ras^{V12}-triggered YAP nuclear translocation and subsequently YAP-targeted gene expression. However, verteporfin, a YAP/TEAD binding inhibitor, failed to prevent Ha-Ras^{V12}-induced multilayer cellular aggregates. Overexpression of Cav1 inhibited Ha-Ras^{V12}-induced YAP activation and multicellular cell aggregates, whereas knockdown of Cav1 in MDCK cells resulted in activation of YAP, but not cellular aggregates and cellular transformation. Activities of Rac and RhoA, both associated with cell extrusion, were increased in Ha-Ras^{V12}-overexpressed MK4 cells. EHT1864 (Rac inhibitor) abolished multilayer cellular aggregates in Ha-Ras^{V12}-overexpressed MK4 cells, whereas Y27632 (ROCK inhibitor) induced multilayer cellular aggregates in MK4 cells. However, neither EHT1864 nor Y27632 inhibited Ha-Ras^{V12}-induced YAP nuclear localization. Finally, Ha-Ras^{V12}-overexpression induced a marked increase in exosome secretion and cell softening, which preceded cell clumping.
Summary/conclusion: Taken these data together, our results indicate that Cav-1 downregulation is required for Ha-Ras^{V12}-induced YAP activation and cellular transformation. However, only Cav1 downregulation and Rac activation, but not YAP activation, are required for Ha-Ras^{V12}-induced multilayer cellular aggregate.
Funding: This work was partially funded by MOST 107-3017-F-006-002 to MJ Tang.

LBF01.04

Longitudinal analysis of serum-derived extracellular RNA for monitoring of treatment response to targeted therapy in glioblastomas

Leonora Balaj[a], Rob Kitchen[b], Bob Carter[c], Xandra Breakefield[c] and Johan Skog[d]

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Introduction: We performed longitudinal whole-transcriptome profiling of serum exosomes from patients suffering from recurrent glioblastoma (GBM) enrolled in a clinical trial to assess response to Dacomitinib, a second-generation irreversible EGFR tyrosine kinase inhibitor. All patients had failed standard of care therapy and had tumours amplified for EGFR. They underwent daily oral administration of Dacomitinib and blood serum samples were collected immediately prior to first treatment and monthly thereafter.
Methods: Deep sequencing of exosomal RNA (exRNA), derived from just 2 ml of patient serum, revealed robust signatures of treatment response, as defined by >6-month progression-free survival.
Results: Non-responders were found to have significantly elevated levels of a number of transcripts including colony stimulating factor 1, which regulates the proliferation, differentiation, and survival of macrophages and microglia. We further identified robust signatures of treatment response in post-treatment serum samples, including the suppression of DNA methyltransferase 3 alpha, an important player in DNA methyl transfer for de-novo methylation, as well as Adenosine A2B receptor, a member of the G protein-coupled receptor superfamily which is overexpressed in a variety of cancers and has been shown to play a role in tumour progression via increased angiogenesis and metastasis. Furthermore, we identified general decreases in oncogene abundance following Dacomitinib treatment, including tumour protein p53 and Ovo-like transcriptional repressor 1, a zinc finger-containing transcription factor, shown to be a critical inducer of epithelial-to-mesenchymal transition in cancer. Finally, in comparison to healthy control serum, we find hundreds of transcripts exhibiting differential abundance in pretreatment GBM patients that may serve as general non-invasive biomarkers for this devastating disease.
Summary/conclusion: This study is unique because it represents the first longitudinal profiling of the exosomal transcriptome in a cohort of genomically selected GBM patients. These findings are a tantalizing step

towards exosome-based biomarkers for the detection of GBM, as well as patient stratification and monitoring.
Funding: CA069246 CA230697 TR000931

LBF01.05

Lung cancer exosome specific protein 1 (LESP-1) is increased in the plasma of non-small cell lung cancer patients

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Introduction: Lung cancer remains to be the leading cause of cancer-related mortality worldwide. Finding new non-invasive biomarkers for lung cancer is still a significant challenge. Exosomes are endosome-derived, nano-sized (30–150 nm), extracellular microvesicles released from many cell types, and that play a key role for in cell-to-cell communication. Use of exosomes as biomarkers in of lung cancer, in a liquid biopsy, is a rising emerging field in nanotechnology in a liquid biopsy. This research work focused on identifying exosome-specific proteins (LESP) of in non-small cell lung cancer (NSCLC) by using proteomics and assessed their concentration efficacy within exosomes derived from the plasma of normal and NSCLC patients.
Methods: Proteomics analysis was performed to investigate lung cancer-specific proteins within exosomes isolated from five NSCLC (H522, A549, H1299, H1650, PC9) and one normal lung alveolar cell lines (Human pulmonary alveolar epithelial cell), using size exclusion chromatography. We then isolated plasma exosomes from healthy controls and NSCLC patients (17 controls and 54 patients) using dual size exclusion chromatography. ELISA and Western blot were utilized to validate the proteomic results in NSCLC patients and compare with healthy controls.
Results: Using proteomics analysis, we identified LESP-1 in the exosomes from NSCLC cells, but not in those from normal cells. LESP-1 concentration was higher in lung cancer patients compared to the healthy controls ($p < .01$), and increased according to the grade of lung cancer, in peripheral blood ($p < .01$). Moreover, Western blot results confirmed the increase in LESP-1 in NSCLC patients, with band intensity increasing with the grade of lung cancer, compared to healthy controls.
Summary/conclusion: LESP-1 in exosomes was highly expressed in the blood plasma of lung cancer patients, which suggests that LESP-1 could serve as a potential biomarker for diagnosis of NSCLC.
Funding: This research was supported by the Korea Health Technology R&D Project (No. HR14C0007) funded by the Ministry of Health & Welfare, Republic of Korea.

LBF01.06

Chloride intracellular channel protein 4 (CLIC4) is a serological cancer biomarker released from tumour epithelial cells via extracellular vesicles and required for metastasis

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Introduction: Chloride intracellular channel protein 4 (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 acting as a tumour promoter. Recent reports indicate that CLIC4 is detected in the circulation of cancer patients serving as possible biomarker and has been detected in extracellular vesicles (EVs).
Methods: EVs from multiple sources 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 prototypical markers and CLIC4 were analysed by immunoblot and by tissue staining.
Results: CLIC4 was present in EVs released from primary normal and multiple breast tumour cell lines and increased in EVs from TGF-β-induced myofibroblasts. *In vivo*, in two different orthotopic syngeneic mouse breast cancer models, CLIC4 levels in EVs isolated from plasma increased with tumour burden and lung metastatic load. Moreover, CLIC4 levels in EVs isolated from plasma of breast cancer patient was elevated when compared to healthy age and race matched controls. To dissect the contribution of stromal vs tumour epithelial compartments as the source of the CLIC4-high EVs, CLIC4 was either deleted in tumour cells lines by CRISPR/Cas9 or CLIC4 KO females were implanted CLIC4 WT tumour cells. CLIC4 is reduced in

circulating EVs from CLIC4 KO tumour bearing mice when compared to WT and it is present in circulating EVs from CLIC4 KO females bearing WT tumours, indicating that the major contribution of CLIC4 into circulation is from tumour epithelium. Additionally, CLIC4 KO females display no difference in primary tumour size and a significant reduction in both size and number of lung metastases.
Summary/conclusion: 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. The low lung metastasis frequency in CLIC4 KO females may due to a defect in lung tissue to recruit neutrophils and to induce neovasculature.
Funding: National Institutes of Health

LBF01.07

Comparative proteomic analysis of exosomes and whole cells from NSCLC cell lines: focus on gefitinib resistance

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Introduction: Overexpression of epidermal growth factor receptor (EGFR) is a typical feature of approximately 90% of NSCLC patients. EGFR mutations induce excessive activation of tyrosine kinase domain of EGFR, eventually inducing oncogenic alterations. Thus, EGFR has become a therapeutic target for NSCLC patients harbouring activating EGFR mutations with tyrosine kinase inhibitor (TKI) such as gefitinib. However, more than 50% of patients with NSCLC receiving gefitinib showed resistance to gefitinib. Thus, acquired resistance to EGFR TKI is a major challenge in the lung cancer treatment. Although several mechanisms have been attributable to acquired resistance, the information on exosomal studies on EGFR-TKIs resistance of NSCLC is limited.
Methods: In this study, comparative proteomic analysis of exosomes and whole cells from EGFR mutant gefitinib-sensitive NSCLC cell lines (PC9) and gefitinib-resistant cell line (PC9/GR) were conducted by quantitative proteomics. The significant protein expression changes observed in each analysis, and the differences of gefitinib resistance-related proteins from exosomes and whole cells were examined.
Results: Biological processes, molecular functions and cellular components associated with gefitinib resistance and key pathways related with gefitinib resistance have been identified in exosomes and whole cell lysates from PC9 and PC9/GR cells. The results also revealed the similarities and differences between gefitinib-resistance of exosomes and whole cells, through pathway analysis of the core functional proteins.
Summary/conclusion: The results might suggest that functional exosomal proteins secreted from gefitinib resistant lung cancer cells contain specific signatures via horizontal transfer from whole cells of NSCLC
Funding: This work was supported by the Industrial Strategic Technology Development Program (10077559) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea).

LBF01.08

Extracellular vesicles derived from bone marrow stromal cells promote evasion of multiple myeloma cells from natural killer cell antitumour activity

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Introduction: Natural killer (NK) cells are a major component of the antitumour immune response. NK cell dysfunctions have been reported in various haematologic malignancies, including multiple myeloma (MM). In the bone marrow of MM patients, bone marrow stromal cells (BMSCs) interact with MM cells, and also create a permissive microenvironment for MM cell survival and immunosuppression. In this study, we investigated the biological property of the extracellular vesicles (EVs) and EV-miRNAs of BMSCs derived from MM patients (MM-BMSCs), aiming to clarify the involvement of EVs derived from MM-BMSCs in tumour immune microenvironment.
Methods: BM samples were obtained from MM patients (age 68–79, n = 6) in accordance with the Declaration of Helsinki and using protocols approved by the research Ethics Committee of Tokyo Medical University (IRB No. 2648), and BMSCs derived from MM patients (MM-BMSCs) were isolated by the classical adhesion method. BMSCs from healthy donors (Normal-BMSCs) were purchased from Lonza Inc. The EVs were isolated from conditioned medium of BMSCs using Exoquick-TC Reagent (System Biosciences). EV-miRNA profiling was done using a TaqMan low-density array (Applied Biosystems). A rapid flow cytometry assay for quantifying target cell killing after CellVue (Sigma)-labelled MM cell lines (RPMI8226, U266) prior to co-incubation with NK cells (Biotherapy Institute of Japan, Inc.).
Results: There were no significant differences in size and amount of EVs among Normal-BMSCs and MM-

BMSCs. We found that the expression of some miRNAs, including miR-146a, in the EVs derived from MM-BMSCs were higher than that of Normal-BMSCs. Furthermore, miR-146a in MM-BMSC-EVs was significantly up-regulated by co-culturing with MM cell lines (RPMI8226, U266). Functionally, when the miR-146a-enriched MM-BMSC-EVs was added to the NK cell-MM cell co-culture system, RPMI8226 become less sensitive to NK cell killing compared with adding the Normal-BMSC-EVs.

Summary/conclusion: These results indicated that MM-BMSC-EVs, including miR-146a, may function as immune regulator to inhibit NK cell function against tumours, therefore providing a new therapeutic target for tumour treatment.

LBF01.09

Exosomal long noncoding RNA NBR2 facilitates the immunosuppression of MDSCs by enhancing the phosphorylation of signal transducers and activators of transcription 3

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Introduction: The immunosuppression induced by myeloid-derived suppressor cells (MDSCs) plays a crucial role in tumour escape. And long noncoding RNA (lncRNA) has been indicated as a core regulator of tumour immunity. However, the effect and regulatory mechanism of lncRNA on the immunosuppressive function of MDSCs remain unclear. Exosomes are cell-derived vesicles which are present in high abundance in the tumour microenvironment where they transfer information between cells.

Methods: Ultracentrifugation was used to isolate exosomes from cancer cells. MDSCs and T cells were sorted from the spleen of tumour-bearing mice and wild type mice, respectively, with immunomagnetic beads. CFSE was performed to estimate the influence of MDSCs on the proliferation of T cells. And real-time fluorescence quantification PCR (qRT-PCR) was used to detect the expression of lncRNA NBR2, while western-blot was used to confirm the phosphorylation of signal transducers and activators of transcription 3 (STAT3).

Results: Herein, we found that tumour-derived exosomes (TEXs) could enhance the development and immunosuppression of MDSCs. Furthermore, it was indicated that the regulation of TEXs to the development and immunosuppression of MDSCs depending on the transportation of lncRNA NBR2 from cancer cells to MDSCs. Besides that, lncRNA NBR2 conveyed by TEXs induced the development and suppressive function of MDSCs by interacting with STAT3 and enhancing its phosphorylation.

Summary/conclusion: Thus, this present study indicates that exosomal lncRNA NBR2 can increase the immunosuppression of MDSCs by regulating the phosphorylation of STAT3, which will provide sufficient scientific basis for the immunotherapy of cancer.

LBF01.10

In vivo imaging of natural killer cells labelled with fluorophore-loaded extracellular vesicle mimetics

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Introduction: In the field of cancer immunotherapy, *in-vivo* biodistribution of immunotherapeutic moiety has emerged as important issue as well as its therapeutic efficacy. This is because it plays an important role in assessing the pharmacokinetic aspects associated with the bio-toxicity of the immunotherapeutic moieties injected *in vivo* and evaluating the therapeutic effects associated with homing to lesion sites. Natural killer (NK) cells have non-specific antitumour activity, and have been employed to treat tumours. Unlike other immune cells, NK cells cannot perform phagocytosis sufficiently, so it is difficult to label NK cells with imaging materials such as nanoparticles. Difficulty in labelling NK cells makes it difficult to validate the distribution and antitumour activity of NK cells *in vivo*.

Methods: In this study, we tried to develop NK cell labelling technology using exosome mimetics, based on the fact that exosome mimetics can deliver their cargos to target cells via receptor-mediated endocytosis. We analysed cell adhesion molecules that were overexpressed in NK cells and produced the cell line that overexpress them using cell transformation techniques. We also labelled NK cells with exosome-mimetic nanovesicles loaded with magnetic nanoparticles and fluorophores, and evaluated biomedical imaging and therapeutic effects of the NK cells using mouse tumour models.

Results: We analysed cell adhesion molecules expressed in NK cells and constructed cell lines that overexpress counter receptors. We succeeded in labelling NK cells with a fluorophore-loaded exosome mimetics and also quantitatively evaluated the

biomedical imaging and therapeutic effects of the labelled NK cells.
Summary/conclusion: We produced and characterized NK cell-targeting exosome-mimetic nanovesicles. Exosome mimetics-based cell labelling technology developed in this study will overcome the limitations of current technology and can be widely applied to *in vivo* image-tracking of immune cells in cancer immunotherapy.

LBF01.11

Comparison of MMP-13-containing extracellular vesicles with metastatic ability in human osteosarcoma cells

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Introduction: Osteosarcoma commonly develops from bone and mainly affects children and adolescents. Although therapy for primary osteosarcoma, such as adjuvant chemotherapy combined with surgical wide resection, is being improved, 30–40% of osteosarcoma patients die of lung metastasis. Therefore, it is important to elucidate the mechanism of lung metastasis to establish specific new therapies based on the mechanism. We previously reported that the down-regulation of miR-143 promotes cellular invasion of 143B cells, a human osteosarcoma cell line, and that intravenous injection of miR-143 significantly suppresses lung metastasis of osteosarcoma cells in mice. In addition, matrix metalloproteinase-13 (MMP-13) was identified as one of the miR-143 target genes, and knockdown of MMP-13 was able to suppress the invasion of 143B (metastatic osteosarcoma cell line) cells *in vitro*.
Methods: These data motivated us to examine whether MMP-13 concentration in extracellular vesicles (EVs) secreted by 143B was higher than in that secreted by HOS (non-metastatic cell line). In this study, we examined the number of secreted EVs and MMP-13 concentration in the EVs of two human osteosarcoma cell lines-143B and HOS.
Results: The number of EVs secreted by 143B was four times higher than those secreted by HOS. Moreover, Western blot analysis revealed that MMP-13 concentration per 3 μg of EVs was increased 2.5 times in EVs derived from 143B in comparison to those derived from HOS.
Summary/conclusion: These data suggest that the number of secreted EVs and/or the concentration of MMP-13 in EVs play an important role in the metastatic ability of human osteosarcoma cells.

LBF01.13

Exosomal long noncoding RNA NBR2 induces the autophagy of lung cancer cells by interacting with high-mobility group box 1

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Introduction: Lung cancer has become the leading cause of disease-related death worldwide. It has been confirmed that high-mobility group box 1 (HMGB1) is closely correlated with the progression of lung cancer. However, it still remains unclear about the accurate mechanisms of regulating the expression and secretion of HMGB1 in lung cancer cells. Exosomes are cell-derived vesicles which are present in high abundance in the tumour microenvironment where they transfer information between cells.
Methods: Exosomes from cultivate supernatant of lung cancer cells were isolated with ultracentrifugation. Western-blot and immunofluorescence were performed to confirm the expression of HMGB1 in lung cancer cells, and ELISA was used to detect the secreted HMGB1. The expression of long noncoding RNA (lncRNA) NBR2 was detected with real-time fluorescence quantitative fluorescence (qRT-PCR). Western-blot and transmission electron microscope were used to make sure the autophagy of lung cancer cells.
Results: Herein, we demonstrated that exosomes from lung cancer cells could promote the both the expression and secretion of HMGB1, and therefore induce the autophagy of lung cancer cells. Besides that, it was also demonstrated that exosomes from lung cancer cells promoted the expression and release of HMGB1 by conveying lncRNA NBR2 which could interact with HMGB1 protein and enhance its stability. Moreover, high level of HMGB1 facilitated the autophagy of lung cancer cells via activating RAGE-Erk1/2 pathway, and accelerated the progression of lung cancer.
Summary/conclusion: Taken together, our study indicates that exosomal lncRNA NBR2 induces the autophagy of lung cancer cells and accelerates the tumour progression by interacting with HMGB1.

LBF01.14

Morphology of tissue disruption at the sites of high-grade tumour

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Introduction: Invasive cancers originating from diverse organs like breast, ovary and lung metastasize to distant sites. However, the structural changes at the primary site of these high-grade tumours have not been well characterized. In this observational study using images of hematoxylin-eosin stained human tumour tissues, it is reported that intriguingly, the morphology of the tissues at the metastatic front or at the primary site appears similar, irrespective of the organ of origin of the primary tumour. These structures appear as a bulb-like projection, emanating from the tumour cell membrane. Mostly, they appear similar as a ball and stick bulge. Although structures are not exactly similar in their dimension, there is a conserved bulb like morphological protrusion in all tumours. Earlier studies have described in *in vitro* or mouse models of similar structures as invadopodia, invadosome or podosome, but their morphology has not been well characterized and examined in detail.

Methods: Web pathology website of H&E-stained human tumour slides were used. Cases have been studied based on the H&E-stained slides and on their diagnosis available for each case. Each organ and tissue specific tumours were selected after systematically browsing all seminars. Control cases were chosen based on their diagnosis notes as non-invasive benign tumour condition. In this study, image J was used for its different potentials. It was used to prepare the figures to show boundary, measure the perimeter, making efficient colour contrast figures.

Results: The preliminary observations reported in this study suggest that there may be excessive force acting in the periphery of the cell, which likely leads to this cytosol enclosed membrane protrusions, likely to contain the nuclei. Some of these protrusions have nuclei and others do not. Also, there is clear evidence of enhanced membrane synthesis in high-grade tumour tissues.

Summary/conclusion: This study suggests that there is a possible final common pathway of mechanical force generation, likely via cytoskeleton remodelling, which may arise irrespective of organ-specific genomic modulation related to the primary disease process. These observations provide visual evidence of epithelial-mesenchymal transition during tumour metastasis.

Funding: none

Late breaking- EVs and physiology
Chairs: Elie Beit-Yannai; Benedikt Kirchner
Location: Level 3, Hall A

15:30–16:30

LBF02.01=OWP1.10

Type-2 transglutaminase affects calcium homeostasis in neurons and is released in association with astrocytes-derived exosomes

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Introduction: Type-2 transglutaminase (TG2) has been linked to calcium (Ca2+) dysregulation in conditions such as neurodegeneration. Recent evidences suggest that extracellular vesicles (EVs) contribute to the onset and progression of neurological diseases, and we have recently shown that TG2 is a cargo of EVs in biological fluids. Here, we hypothesise that TG2 could be released by EVs, interact with neurons and affect neuronal Ca2 + homeostasis.

Methods: Primary hippocampal neurons were established from E18 rat embryos. Extracellular TG2 was modulated in neurons either by lipofectamine transfection of a TG2-EGFP construct or by addition of purified TG2. Intracellular Ca2+ concentration ([Ca2+]i) was assessed by live imaging in fura-2/AM-loaded neurons. EVs were isolated from primary astrocytes (60 DIV) by serial centrifugation, characterized by Western blotting (flotillin-2 and alix) and nanoparticle tracking analysis (ZetaView). Experiments to assess TG2 influence on exosomes-to-neural cells interactions, using a Renilla sensor based on miR-146a-5p-transfer, are still ongoing.

Results: Increase of extracellular TG2 levels in neurons induced an influx of extracellular Ca2+ ions, leading to a significant raise in basal [Ca2+]i both in normal conditions ($\Delta F340/380 = 0.126 \pm 0.014$; $N = 23$; $p < 10^{-5}$) and with inhibited synaptic transmission (tetrodotoxin) ($\Delta F340/380 = 0.058 \pm 0.005$; $N = 33$; $p < 10^{-5}$). Nifedipine, a blocker of L-type voltage-operated Ca2+ channels (VOCCs), partially prevented TG2-dependent Ca2+ response (average inhibition 36%; $N = 21$; $p < 10^{-5}$), suggesting that Ca2+ influx may occur through L-type VOCCs. To identify the source of extracellular TG2, we analysed EVs isolated from rat primary astrocytes, previously reported to release TG2 into the matrix especially in inflammatory conditions. TG2 was detected in astrocytic exosomes (but not in ectosomes) only upon LPS stimulus, and not in the EVs-free medium, suggesting that TG2 is a cargo of exosomes during neuroinflammation.

Summary/conclusion: TG2 is externalized through astrocyte-derived exosomes upon neuroinflammatory stimuli. Extracellular TG2 mediates the opening of L-type VOCCs in neurons and sets basal [Ca2+]i at higher levels, which could have a significant impact on neuronal activity in neuroinflammation.

Funding: John Turland PhD bursary (NTU) and IBRO travel fund.

LBF02.02=OWP1.12

Plasma exosomes regulate proliferation and migration of vascular smooth muscle cells

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Introduction: We previously reported that systolic blood pressure in spontaneously hypertensive rats, an animal model of essential hypertension, was partly modulated by circulating exosomes (*BBRC* 2018). Vascular wall remodelling regulated by proliferation and migration of vascular smooth muscle cells (VSMCs) mediates development of hypertension. We aimed to clarify the effects of plasma exosomes derived from SHR and control Wistar Kyoto Rats (WKY) on proliferation and migration of VSMCs.

Methods: Exosomes were isolated from rat plasma by an ultracentrifuge method, and identified through measurement of particle size distribution by a tunable resistance pulse sensing. For exploring exosome internalization in VSMCs, the isolated exosomes were labelled with PKH67 dye and observed by a fluorescence microscopy. Proliferation and migration of SMCs were determined by a bromodeoxyuridine incorporation and Boyden chamber assay, respectively. Actin cytoskeleton was visualized by a rhodamine-phalloidin staining. Expression of protein and microRNA in exosomes was determined by Western blotting and microarray, respectively.

Results: There was no difference in size and concentration of plasma exosomes between WKY and SHR. Exosomes were incorporated into VSMCs, while the internalization of SHR exosomes was significantly lower than WKY exosomes. Both WKY and SHR exosomes similarly stimulated proliferation, migration and cytoskeletal changes such as formation of filopodia and lamellipodia in VSMCs. Heparin, an inhibitor of exosome internalization, completely blocked the migration and proliferation. Protein expression of CD9 and CD63, an exosomal marker, was significantly higher in exosomes from WKY than SHR. The expression of several microRNAs in SHR exosomes changed compared with WKY exosomes.

Summary/conclusion: These results suggest that plasma exosomes play physiological, but not pathological, role on VSMCs irrespective of their origin (normotensive or hypertensive rats). Further research is required for determining whether the changes in molecular profiles of circulating exosomes mediate the development of high blood pressure in SHR.

LBF02.03

Exosomes containing HIV protein Nef reorganize lipid rafts inducing inflammatory responses of immune cells and increasing their susceptibility to HIV infection

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Introduction: HIV disease is associated with co-morbidities, which persist even after successful application of antiretroviral therapy. One important factor responsible for these effects is exosomes containing the main HIV pathogenic factor, Nef, which can be found in blood of HIV subjects with undetectable HIV load. In this study, we investigated the effect of Nef-containing exosomes on uninfected macrophages and T cells.

Methods: Exosomes were purified by differential centrifugation from HEK293 cells transfected with Nef-expressing or empty vector, monocyte-derived human macrophages infected with Nef-positive or Nef-deficient HIV-1, or plasma of uninfected subjects or patients infected with wild-type or Nef-deficient HIV-1. Exosomes were adjusted by protein content and added to cells at 0.2 ng/ml of Nef protein. Mice were injected with Nef exosomes IP (2 µg per injection) 3 times a week for 2 weeks.

Results: Exosomes containing HIV protein Nef (exNef) are internalized by macrophages altering cholesterol metabolism and causing sharp increase in the abundance of lipid rafts through reduced activation of small GTPase Cdc42 and decreased actin polymerization. ExNef increased susceptibility of MDM to fusion with HIV. These changes resulted in activation of NLRP3 inflammasome and increased secretion of pro-inflammatory cytokines. The effects of exNef were reversed by overexpression of a constitutively active mutant of Cdc42. The findings were validated with exosomes produced by HIV-infected cells and with exosomes isolated from plasma of HIV-infected subjects, but not from ΔNef-HIV-infected subjects. Mice injected with exNef displayed altered cholesterol metabolism, monocytosis, increased raft abundance and augmented inflammation.

Summary/Conclusion: Nef containing exosomes potentiate pro-inflammatory responses by impairing cholesterol metabolism and reorganizing lipid rafts, increasing cell susceptibility to HIV infection and contributing to co-morbidities of HIV disease.

Funding: Grants from NIH (HL131473, AI055019), American Heart Association (17GRNT33630163), and NHMRC (GNT1036352).

LBF02.04

Exosomes derived from human mesenchymal stem cells ameliorates autistic-like behaviours in BTBR and Shank3 mice models of autism

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Introduction: Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by three core symptoms that include severe impairment of social interaction and communication skills, increased repetitive behaviours and cognitive inflexibility. Rate of ASD is steadily increasing in children over the past several years, with no effective treatment.

Methods: BTBR and Shank3 are accepted mouse models used for evaluating autistic-like behaviours as it presents all core symptoms and genetic human mutation of ASD. We have previously shown that transplantation of human bone marrow mesenchymal stem cells (MSCs) to the lateral ventricles of BTBR mice results in long lasting improvement in their autistic behavioural phenotypes. Recent studies point of exosomes as the main mediators of the therapeutic effect of MSCs.

Results: Here we show that intranasal administration of exosomes derived from bone marrow or adipose tissue MSCs, ameliorate autistic-like behaviour in

BTBR and Shank3 mice. Including significant increase of social interaction and ultrasonic vocalizations, reduced repetitive behaviours and improve maternal behaviours of pup retrieval. No negative safety symptoms were detected following exosomes intranasal treatments in mice.
Summary/conclusion: The beneficial effects of the exosomes treatment in mice models may be translated to a novel, easy to administer, therapeutic strategy to reduce the symptoms of ASD.
Funding: Partially by Stem Cell Medicine LTD and Kamin

LBF02.05

The use of artificially produced bacterial vesicles as an immunotherapeutic vaccine against Pseudomonas aeruginosa pneumonia

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Introduction: *Pseudomonas aeruginosa* is an opportunistic pathogen which is involved in pneumonia and cystic fibrosis. Immunization with outer membrane vesicles (OMVs), which has naturally budded off from bacteria, is an evolving field in infectious diseases due to their highly immunogenic properties. However, OMVs are difficult to produce naturally in large quantities with high purity. This study aims to develop an artificial OMVs (aOMVs) isolation method, and to investigate the protective effects of aOMVs against *P. aeruginosa*-induced pneumonia.
Methods: Outer membranes were obtained from *P. aeruginosa* by lysozyme and detergent treatment, followed by ionic stress and applied mild energy to generate aOMVs. The yield and purity of aOMVs were analysed by nanoparticle tracking analysis and transmission electron microscopy. The protein and RNA contents were examined by label-free quantitative mass spectrometry and bioanalyzer. Inflammation was evaluated in lung macrophages by measuring cytokine release. Naturally produced OMVs or aOMVs were weekly injected in mice for 3 weeks, and then blood and spleen were obtained for antibody titer and splenocyte cytokine study. Lung inflammation by *P. aeruginosa* challenge was assessed in H&E stained lungs.
Results: The aOMVs were isolated in higher yield (fivefold) compared to OMVs. They had similar spherical shape and size as OMVs, but had better purity. Outer membrane components were more enriched in aOMVs, and cytosolic protein and RNA contents were almost completely removed. Especially, aOMVs were observed to induce less inflammation in macrophages compared to OMVs. In addition, immunization with aOMVs induced strong IgG antibody response to the bacterial proteins, and a greater increase in IFN-gamma from CD4+ T cells compared to OMVs. Moreover, aOMV-immunized mice showed dramatically reduced lung inflammation caused by bacterial challenge.
Summary/conclusion: This study shows that aOMVs can be produced in a large amount with high purity, and have protective effect against *P. aeruginosa*-induced pneumonia through a balanced humoral and cellular immune response, suggesting that aOMVs may be novel bacterial vesicle-mimetics to clinically applicable to infectious diseases.
Funding: This work was supported by Swedish Heart-Lung Foundation (20150588).

LBF02.06

Herpesvirus infection of infant tonsil mucosal epithelia containing intravesicular Human Immunodeficiency Virus induces release of HIV from epithelial cells

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Introduction: Mother-to-child transmission (MTCT) of HIV is an important pathway for the spread of the virus from mother to child; however, the molecular mechanisms of HIV MTCT are poorly understood. Our recent work showed that >90% of virions internalized into polarized infant tonsil epithelium were sequestered, that is, trapped in the endosomes, including multivesicular bodies (MVBs) and vacuoles of epithelial cells, for up to 9 days. The primary goal of this work was to investigate the role of common oral viral pathogens herpes simplex virus-1 (HSV-1), human cytomegalovirus (HCMV), and Epstein-Barr virus (EBV) in the release of endosomal HIV, which may play a role in HIV MTCT.
Methods: Polarized tonsil epithelial cells were incubated with HIV-1. After 4 h, the extracellular virus was removed, and cells were maintained for 3 days. Cells were then infected with HSV-1, HCMV, and EBV. AP and BL medium was independently collected after herpesvirus infection and HIV release was examined by p24 ELISA assay.
Results: Our data showed that the infection of HSV-1, HCMV and EBV in tonsil epithelial cells containing intravesicular HIV-1 led to the release of HIV virions, which were infectious for peripheral blood mononuclear cells. HIV release was correlated with the reduction

of TER in HSV-1-, HCMV- and EBV-infected polarized epithelial cells; that is, herpesvirus-induced depolarization of epithelial cells was critical for HIV release. HSV-1 and HCMV infection in tonsil epithelial cells substantially increased the expression of GTPases Rab27a and Rab27b, which may regulate the movement of MVBs and vacuoles to the plasma membrane and their fusion with the epithelial cell membrane.

Summary/conclusion: HSV-1- and HCMV-induced activation of Rab27a and Rab27b in epithelial cells containing intravesicular HIV may promote virus release, that is, exocytosis of virions. HSV-1-, HCMV- and EBV-induced depolarization of tonsil epithelial cells also may play critical in the release of endosomal HIV. Herpesvirus interaction with infant tonsil epithelial cells containing HIV may lead to the release and spread of HIV into CD4+T lymphocytes, macrophages and Langerhans/dendritic cells, leading to HIV MTCT.

Funding: R01DE028129, NATIONAL INSTITUTE OF DENTAL & CRANIOFACIAL RESEARCH

LBF02.07

Syntenin regulates Hepatitis C virus sensitivity to neutralizing antibody by promoting E2 secretion through exosomes

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Introduction: Hepatitis C virus (HCV) is a major cause of chronic liver disease, infecting approximately 71 million people worldwide. Assembly of infectious HCV particles involves host lipoproteins, giving rise to unique lipo-viro-particles (LVPs), but proteome studies suggest that additional cellular proteins are associated with HCV virions or other particles containing the viral envelope glycoprotein E2. Many of these host cell proteins are common markers of exosomes, most notably the intracellular adaptor protein syntenin required exosome biogenesis. These observations suggest that E2 might be a component of both LVPs and exosomes produced from HCV infected cells.

Methods: Using HCVcc in both hepatoma cells and primary human hepatocytes (PHHs), we studied biogenesis and function of E2-coated exosomes.

Results: Knockout of syntenin had negligible impact on HCV replication and virus production whereas ectopic expression of syntenin at physiological level reduced intracellular E2 abundance concomitant with increased secretion of E2-coated exosomes. Importantly, cells expressing syntenin and HCV structural proteins efficiently released exosomes containing E2 but lacking the core protein. Furthermore, infectivity of HCV released from syntenin expressing hepatoma cell and PHHs was more resistant to neutralization by E2-specific antibodies and chronic-phase patient serum. Last, high E2/syntenin levels in sera correlates to lower serum neutralization capability.

Summary/conclusion: E2- and syntenin-containing exosomes is a major type of particles released from cells high expressing syntenin. Efficient production of E2-coated exosomes in hepatoma cells and PHHs renders HCV infectivity less susceptible to antibody neutralization.

Funding: This work was supported by from the strategic priority research program of the Chinese Academy of Sciences (XDB29010000), the National Science and Technology Major Project of the Ministry of Science and Technology of China (2015CB554300 and 2016YFC1200400) and the National Nature Science Foundation of China (81761138046). Work by R.B. was supported by the Deutsche Forschungsgemeinschaft, collaborative research center (TRR) 179, TP9.

LBF02.08

Lipidomics profiles of plasma microvesicles differ in experimental cerebral malaria, compared to malaria without neurological complications

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Introduction: Cerebral malaria (CM), a fatal complication of Plasmodium infection affecting children in sub-Saharan Africa and adults in South-East Asia, results from incompletely understood pathogenetic mechanisms, which include sequestration of infected erythrocytes, cytokine overproduction, accumulation of inflammatory cells, and excessive release of microvesicles (MV). Plasma MV levels are elevated in CM patients and in the experimental mouse model. Here, MV lipidomics profile was studied in relation to the development of cerebral complications.

Methods: Plasma MV was enriched using differential centrifugation (El-Assaad 2014). Lipids were extracted according to Matyash et al. (2008), loaded on a C30 Acclaim column using a Vanquish liquid chromatography (LC) system and analysed using a Fusion mass spectrometer (MS). LipidSearch software was used for lipid species annotation and quantification.

Results: We compared lipid profiles in circulating MV purified from CBA mice with P. berghei ANKA (PbA), which causes CM, to those from *P. yoelii* (Py), which does not. Plasma MV produced at the time of CM dramatically differed from those from non-CM mice, in spite of identical levels of parasitaemia: using high-resolution LCMS, we identified over 200 lipid species within 12 lipid classes. Total phosphatidylethanolamine (PE) levels were significantly higher in MV from PbA mice compared to those from uninfected control and Py. Using fragmentation MS, we identified that this PE increase is due at least in part to PE (16:0_22:6), PE (18:0_22:6) and PE (18:1_22:6) species identified in PbA vs Py and uninfected control. Total phosphatidylserine (PS) was significantly higher in both PbA and Py compared to uninfected control. Conversely total lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) were significantly lower in PbA compared to uninfected mice, while they were unchanged in Py MV.
Summary/conclusion: These results suggest, for the time, that experimental CM is characterized by specific changes in lipid composition of circulating MV, pointing towards PE subsets, LPC and LPE as potential important players in CM pathogenesis.
Funding: NHMRC Project grant APP1099920 to GG.

LBF02.09

Compound extracted from cinnamomum osmophloeum leaves reduced exosomes release from hepG2 cells

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Introduction: Cinnamomum osmophloeum belongs to the genus of Cinnamon, the same genus as the species used for commercially sold cinnamon. Compounds of the extracted Cinnamomum osmophloeum leaves have good potential to be developed into new drugs. Further, usage of the leaves of the tree is much more sustainable and cost effective than the bark. ABL006 is a major compound isolated from Cinnamomum osmophloeum that previously known for insulin mimetick effect. For fear of side effect of pro-inflammatory effect to the central nervous system, we tested using proteomic approach to study differential protein expression after ABL006 treatment in astrocytic cells.
Methods: We used dimethyl labelling on the peptide level and LC-MS/MS to select differentially expressed proteins. The selection criterion was based on significant up- or down-regulation in both biological samples.
Results: We were able to quantitate 13,013 peptides, which corresponds to 1264 proteins from two biological replicates. Thirty-two differentially expressed proteins were shortlisted, among them some are nuclear protein and protein relevant to lipid metabolism. Further pursuing this, we treat hepG2 with ABL006, and study the differential protein expression in the conditioned medium, hoping to understand further the lipid regulating action of ABL006. The differentially expressed proteins between treated and non-treated were short-listed to 33 proteins. These proteins were checked against the 100 top expressing proteins secreted by the exosomes (Exocarta, http://exocarta.org/index.html). Out of 33 most significantly regulated proteins, 8 were exosomal markers, and almost all were down-regulated upon ABL006 treatment.
Summary/conclusion: This suggested that exosomes release from hepG2 is reduced upon ABL006 treatment.
Funding: MOST 107-2632-B-324-001.

LBF02.10

Placental cells function as environmental sensors that respond to changes in the extracellular milieu via extracellular vesicles

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Introduction: Placenta-derived extracellular vesicles (PdEVs) are present in maternal circulation as early as 6 weeks of gestation. Changes in the concentration of PdEVs are found in gestational diabetes, preeclampsia and preterm birth. The aim of this study was to characterize the release and biogenesis of EVs from placental cells in response to extracellular glucose, insulin, lipopolysaccharide (LPS) and tumour necrosis factor a (TNF-a) *in vitro*.
Methods: Bewo cells were used as a placental model. Cells were incubated with forskolin for 24 h to stimulate syncytium formation *in vitro*. After syncytialization, cells were incubated in the presence of forskolin with D-glucose (5 mM or 25 mM), insulin (1 nM), LPS (0–10 μg/ml) and TNF-a (0–20 ng/ml) for 48 h. EVs were isolated from cell-conditioned media by differential centrifugation and characterized by their size distribution, protein abundance and morphology using

nanoparticle tracking analysis, Western blot and electron microscopy, respectively. The effect of the extracellular milieu on the release of PdEVs was evaluated in four different subpopulations according to size; <50, 50–150, 150–200 and >200 nm.

Results: Differential changes in the release of PdEVs subpopulations in response to glucose, insulin, LPS and TNF-a were observed. High glucose induced the release of EVs <50 nm, and >200 nm although this effect was abolished by insulin. High glucose and insulin decreased the release of EVs 150–200 nm and EVs 50–150 nm, respectively. The effect of LPS on the release of PdEVs was size-dependent with the greatest effect on EVs of >200 nm. Finally, TNF-a increased the release of EVs in size and concentration-dependent manner with a maximum effect on EVs >200 nm and 2 ng/ml. Changes in the release of exosomes were associated with a differential abundance of proteins associated with ESCRT machinery.

Summary/conclusion: The effect of the extracellular milieu on PdEVs release may be recapitulated and is of clinical relevance *in vivo* in association with hyperglycaemia (glucose and insulin), infection (LPS) and inflammatory (TNF-a) conditions.

Funding: Lions Medical Research Foundation, National Health and Medical Research Council (NHMRC; 1114013), Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1170809), and CONICYT PFCHA/DOCTORADO BECAS CHILE/ 2018-72190513

LBF02.11

Association of cytokines with circulating populations of extracellular vesicles at early gestation

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Introduction: Cytokines have several roles across gestation, including implantation, placentation and immune response, which are all essential for the continuation of pregnancy. The aim of this study was to isolate and characterize different populations of extracellular vesicles (EVs) from maternal plasma at the first trimester of pregnancy, and quantify the levels of interleukin 10 (IL-10), 6 (IL-6), Interferon gamma (IFN-γ), and tumour necrosis factor a (TNF-a), associated with EVs.

Methods: Plasma samples were collected from pregnant women during the first trimester of pregnancy (n = 10). EVs were isolated through differential centrifugation, at 2000g for 30 min (pellet 1); 12,000g for 45 min (pellet 2) and at 100,000g for 120 min (pellet 3). The supernatant after the last centrifugation was termed "soluble fractions". EVs were characterized by size distribution, abundance of proteins associated with EVs (i.e. CD63, Flotilin-1 and TSG101), negative control for Grp94, and morphology, according to the recommendations of the International Society of Extracellular Vesicles, using Nanoparticle Tracking Analysis (NTA), Western blot analysis and electron microscopy, respectively. The concentration of IL-10, IL-6, IFN-γ and TNF-a in the EVs and the soluble fractions were established by cytokine array analysis (Bioplex-200).

Results: Specific changes in the levels of cytokines, in the different population of vesicles, and in the soluble fractions were identified. The levels of IL-10, IL-6, IFN-γ and TNF-a were significantly higher ($p < .05$) in the exosome fraction (pellet 3) compared to the values observed in pellet 1 and pellet 2 (macro and microvesicles fractions). The levels of IL-10, IFN-γ and TNF-a were significantly higher ($p < .05$) in the soluble fractions compared with the exosomal fraction. No significant difference in the level of IL-6 in the exosomal and soluble fraction was observed.

Summary/conclusion: This study established that cytokines are packaged within EVs (in which these molecules are protected), suggesting a novel mechanism of action through which cytokines via EVs can lead to distal interactions.

Funding: Lions Medical Research Foundation, National Health and Medical Research Council (NHMRC; 1114013), and Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1170809).

LBF02.12

Mesenchymal stem cell-derived exosomes attenuate inflammation and protect ischemic neuronal damage

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Introduction: Bone marrow mesenchymal stem cells (BM-MSC) are the most extensively used stem cells in tissue engineering due to their easy access, rapid *ex vivo* expansion and poor immunogenicity. MSCs secrete various factors that can modulate a hostile

environment, which is called the paracrine effect. MSCs have a strong capacity for secretion of exosomes which are suspected to participate in paracrine cellular communication.

Methods: We compare the effects of BM-MSC conditioned medium (MSCcm) and MSC-derived exosomes (MSCexo) in neuron-glial cultures as well as in spinal cord injured (SCI) rat model.

Results: We found that both MSCcm and MSCexo were effective in reducing LPS stimulation and oxygen glucose deprivation, an *in vitro* stroke model, damage in neuron-glial cultures. Further comparison of the beneficial effects of MSCcm and MSCexo will be conducted in *in vivo* SCI rats via vascular administration.

Summary/conclusion: This cell-free therapy, avoiding the risks associated with direct MSC transplantation, may improve nerve regrowth and functional recovery after SCI.

Funding: Research grant (V107C-087) from the Taipei Veterans General Hospital in Taiwan, and grants (106-2314-B-075-023 & 107-2314-B-075-021) from the Ministry of Science and Technology in Taiwan.

Symposium Session 19: EV Cargo Profiling
Chairs: Tang-Long Shen, Lei Zheng
Location: Level B1, Lecture Room

16:30–18:00

OF19.01

Distinct extracellular RNA cargo types associate with specific vesicular and non-vesicular RNA carriers across human biofluids

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Introduction: The Extracellular RNA Communication Consortium (ERCC) has developed the ExRNA Atlas, a reference catalogue of exRNAs present in human biofluids. A computational deconvolution analysis identified six RNA cargo types (CT1, CT2, CT3A, CT3B, CT3C, CT4) present across human biofluids represented within the Atlas. Extensive experimental analyses by the ERCC show association of these cargo types with specific vesicular and non-vesicular (lipoprotein, RNP particle) carriers.

Methods: To identify carriers for the six CTs, we performed: cushioned density gradient ultracentrifugation of serum and plasma using the OptiPrep density gradient, RNA-seq, western blot and mass spectrometry analysis of the density fractions; RNA-seq profiling of ultracentrifugation products, of size fractionation using nanoscale deterministic lateral displacement (nano-DLD) arrays; of lipoprotein fractions; and of AGO-1 immuno-pulldowns. We also carried out RNA-seq of a shared pool of human plasma and serum samples processed using 10 widely used RNA isolation methods.

Results: CT1 correlates with RNA-seq profiles of carriers of exosomal size and density (OptiPrep fractions 4–7 that are CD9 and Flotillin positive). CT2 correlates with the exRNA profiles of lipoprotein carriers (HDL, LDL, VLDL, Chylomicron); the carrier shows HDL density (OptiPrep fractions 9–12) and is APOA1 positive. CT3A and B show high miRNA content and correlate with RNA-seq profiles of AGO2 immuno-pulldowns. CT4 correlates with the RNA-seq profiles of both low-density vesicles (OptiPrep fractions 1-3) and HMC-1 cell-line derived vesicles of higher-density. The 10 widely used commercial RNA isolation kits show distinct preferences for specific CT subsets. On average, across all kits, CT4 was captured at highest and CT3B at second-highest relative abundance.

Summary/Conclusion: The heterogeneity of exRNA cargo types exceeds the capabilities of current experimental methods to reproducibly isolate defined carrier subpopulations from human biofluids. While this problem calls for the development of new carrier isolation methods, we have now demonstrated the power of computational deconvolution to complement and enhance current isolation methods and have created the first comprehensive survey of exRNA cargo types and their carriers in human biofluids.

Funding: Common Fund of the NIH (5U54 DA036134).

OF19.02

Heparan sulphate glycosaminoglycans on the extracellular vesicle surface bind a variety of proteins

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Introduction: Cancers develop in complex tissue environments, comprising multiple cell types that contribute to tumour growth, invasion and metastasis. Our group has previously demonstrated that prostate cancer derived EVs mediate the delivery of TGFβ, via heparan sulphate (HS) glycosaminoglycans on the EV surface and stimulate fibroblast to myofibroblast differentiation. Given the potential capacity for HS to bind other "soluble" factors we have herein explored the repertoire of proteins associated vesicular HS.

Methods: EVs were isolated from DU145 prostate cancer cells by differential centrifugation followed by ultra-centrifugation on a sucrose cushion and washed with PBS. Specific removal of Heparan sulphate side chains from the vesicle was performed by enzymatic digestion using heparinase III (HEPIII). Differences in proteins with vs. without digestion were identified by a sensitive multiplex proximity extension assay and select targets validated by ELISA.

Results: Protein profiles identified approximately 60 factors that were significantly differentially expressed on control versus HS-deficient EV's. Some but not all of these have been previously identified as HS-associated factors. Gene ontology analysis points to

functional relationships with angiogenesis, invasion and immune regulation. Using ELISA, we have been able to quantify six selected candidates on wild type vesicles, some of these are lost following HS-digestion. We went on to examine functional consequences of HS-deficiency in relation to cell-uptake, and angiogenic responses.

Summary/Conclusion: These data demonstrate a diverse repertoire of proteins that are bound to the surface of exosomes via HS glycosaminoglycans. We anticipate that removal of EV-associated HS will result in attenuated delivery of multiple factors to recipient cells, and this will have major implications on EV functions and their ability to modulate tissue environments.

Funding: Cancer Research Wales.

OF19.03

Membrane lipid saturation modifies the lipid signature of extracellular vesicles released by HuH7 hepatocarcinoma cells

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Introduction: Extracellular vesicles (EVs) are membrane-limited particles released by cells during physiological and pathological conditions involved in cell communication. An increased ceramide-enriched pro-inflammatory EV release has been demonstrated in *in vitro* lipotoxic conditions and in non-alcoholic steatohepatitis mouse models and patients. So far, lipid profile changes in EVs released under lipotoxic conditions have not been investigated, despite the evidence that EVs shuttle many membrane-derived bioactive lipids playing crucial role in several processes, including inflammation. In this study, we carried out a comprehensive lipidomic analysis of EVs released by HuH7 cells under membrane lipid saturation conditions induced by lipotoxic palmitate (PA) or Δ9 desaturase inhibition (SCD1i). Since membrane lipid saturation induces ER stress, HuH7 cells were also treated with Thapsigargin (Tg), a conventional ER stress inducer, and with oleate (OA), a nontoxic monounsaturated fatty acid.

Methods: EVs were isolated from culture media of HuH7 cells treated for 16 h with fatty acids (400 μM), or Tg (2.5 nM), or SCD1i (CAY 10566, 5 μM). All treatments were performed in serum-free medium containing 0.1% free fatty acids-BSA. EVs were recovered by differential centrifugation and characterized by SEM and immunoblotting. Total lipids from EVs and their parental cells were analysed by LC/MS-MS.

Results: EVs released by HuH7 cells expressed positive markers (Alix and CD63), whose levels were higher in PA-EVs. SEM images showed vesicles with the typical cup-shaped morphology and size, not altered by the various treatments. Lipid composition comparison of EVs released by treated- and control cells showed a higher content of all phospholipid subclasses, in PA-EVs, except for phosphatidic acid and phosphatidylinositol, whereas no differences were observed in EVs released by OA-, Tg- or SCD1i-treated cells. Specific differences have been observed in the levels of numerous phospholipid molecular species in PA-EVs as compared to other treatments.

Summary/Conclusion: In HuH7 cells, the most relevant changes in lipid composition were observed in PA-EVs, even if all treatments, except OA, induced ER stress. These results suggest that the increase of membrane phospholipid saturation is crucial to determine which lipids discarded via EVs.

OF19.04

Deconvolution analysis of small RNAseq data from exRNAs isolated using different methods reveals multiple carrier subclasses and identifies optimal methods for isolation of total and EV-specific exRNA

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Introduction: Reproducibility has been a major challenge in extracellular RNA (exRNA) research both due to low concentration and heterogeneity of exRNA carriers in biofluids, such as EVs, RNPs and LPPs. Lack of knowledge regarding the efficiency/reproducibility of different isolation methods in accessing the exRNAs in different carriers has hindered rational selection of standardized methods.

Methods: Using small RNAseq, we compared the performance of 10 exRNA isolation methods on standardized samples of five biofluids across multiple laboratories. We found that the read depth required to maximize miRNA complexity in each biofluid was different: 1 million in Bile (~200 detected miRNAs), 0.5 million in Cell culture supernatant (~300), 2 million in Plasma/Serum (~450), and 50,000 in Urine (~100). While the miRNA profiles varied greatly among exRNA isolation methods in Plasma, Serum, and Bile, Cell culture supernatant and Urine showed similar profiles for all tested methods.
Results: We performed small RNAseq on purified exRNA carriers from Plasma and Serum; and used the resulting carrier-specific miRNA signatures to computationally deconvolute the miRNA profiles from each of the isolation methods. We found that ExoRNeasy, ME, and Ultracentrifugation purified miRNAs that were predominantly carried in EVs, while Exiqon, ExoQuick, and Norgen isolated both EV- and AGO2+ RNP-associated miRNAs.
Summary/Conclusion: Our studies identified several factors that contribute to difficulties with reproducibility in exRNA studies, like inefficient and variable exRNA isolation for many of the available methods, differences in accessibility of miRNA cargo associated with different carriers among methods, and insufficient sequencing depth. To help investigators select an optimal method, we developed an interactive web-based application, miRDaR, that will provide a ranked list of tested exRNA isolation methods by complexity/expression level and reproducibility, specific to their biofluid and miRNA of interest.
Funding: This study was supported by the Extracellular RNA Communication Consortium funded by the NIH Common Fund.

OF19.05

Novel angiogenic extracellular vesicles induced by StemRegenin1

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Introduction: Aryl hydrocarbon receptor antagonists, such as StemRegenin1 (SR1), have been recently shown to enhance expansion of hematopoietic stem progenitor cells (HSPCs). Our preliminary data showed that SR1 enhances endothelial cells (EC) to promote HSPC expansion possibly via direct and indirect intercellular interactions, including extracellular vesicle (EV) production. However, the direct effect of SR1 on EC biology and EV production is largely unknown.
Methods: Human umbilical vein EC (HUVEC) and HSPC were obtained per approved IRB protocol. EC culture and EC-HSPC *in vitro* co-culture was performed as described previously. EC-EV harvest was collected in serum free medium. The effect of SR1 on HUVEC growth was evaluated by EC-colony-forming cell (EC-CFC) assay. HSPC population analysis was done by FACS. EV was purified by ultracentrifugation. EV proteins were identified by LC-MS/MS. Student t-test was performed with $p < 0.05$ for statistically significance.
Results: HSPC co-cultured with HUVEC in the presence of SR1 results in a ~ 2-fold increase of more primitive HSPC subpopulation compared to the control group. SR1 treated HUVEC leads to a significant >2-fold increase in EC-CFC numbers and >67% increases in the colony diameter. A total of 327 proteins were detected in ECs derived from HUVEC. As a quality control, many commonly reported "EV-enriched" proteins per "ISEV position statement" were identified. A small fraction of proteins recovered from the EV fraction (<2%) is found on EC membrane. Among the 327 proteins, 46 of them showed a significant change with SR1 treatment. Functional annotation by DAVID bioinformatics enrichment tools classified three EV proteins associated with enhanced angiogenesis signalling pathways.
Summary/Conclusion: SR1 differentially regulates angiogenic EV production that associates with increased robustness in endothelial growth and enhanced haematopoiesis. Future investigations on the biological effects of HUVEC EV differentially produced by SR1 are in progress and needed.

OF19.06

Evaluation of circulating extracellular vesicles derived miRNAs as biomarkers of early colon cancer: a comparison with plasma total miRNAs

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Introduction: Early diagnosis of colon cancer (CC) is clinically important, as it can significantly improve patients' survival rate and quality of life. Although the potential role for small extracellular vesicles (sEVs) in early detection of many diseases has been repeatedly mentioned, systematic screening of plasma sEVs derived early CC biomarkers has not yet been reported.

Methods: Plasma sEVs were derived from 15 early stage CC patients and 10 normal controls (NC) and characterized according to MISV2014 standard. The total circulating sEVs derived microRNA (miRNA) expression profile of all participants was investigated by next-generation sequencing (NGS). Selected miRNA candidates were further verified in both plasma-derived sEV miRNA and plasma total miRNA of an independent cohort consisting of 134 participants.

Results: RNA sequencing identified a total number of 95 sEVs miRNA with differential expression between CC and NC, most of which (60/95) was in well accordance with tissue results in the Cancer Genome Atlas (TCGA) dataset. Among those miRNAs, we selected let-7b-3p, miR-139-3p, miR-145-3p, and miR-150-3p for further validation in an independent cohort consisting of 134 participants (58 CC and 76 NC). In the validation cohort, the AUC of 4 individual miRNAs ranged from 0.680 to 0.792. A logistic model combining two miRNA (i.e. let-7b-3p and miR-145-3p) achieved an AUC of 0.901. Adding the 3rd miRNA (miR-139-3p) into this model can further increase the AUC to 0.927. Side by side comparison revealed that sEVs miRNA profile outperformed cell-free plasma miRNA in the diagnosis of early CC.

Summary/Conclusion: Circulating sEVs have a distinct miRNA profile in CC patients, and sEVs derived miRNA could be used as a promising biomarker to detect CC at an early stage.

Funding: This work was supported by grants from the National Natural Science Foundation of China (81702314).

Symposium Session 20: EV Therapeutics II
Chairs: Minh Le; Lucia Languino
Location: Level B1, Hall B

16:30–18:00

OF20.01

Nano-Ghosts: mesenchymal stem cells derived nanoparticles as a novel approach for cartilage regeneration.

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Introduction: Osteoarthritis is the most common inflammatory disease of the joints which is characterized by cartilage degeneration and bony overgrowth. Mesenchymal stem cells (MSCs) play an essential role in inflammation, due to their aptitude to home to inflamed tissues and modulate the process. We designed a new kind of particles termed Nano-Ghosts (NGs), derived from the cytoplasmic membrane of the MSCs. Retaining MSCs' surface properties, NGs are expected to target inflamed tissue and modulate inflammation. In this study, we demonstrate NGs' ability to target human articular chondrocytes (hACs) and cartilage explants while reducing inflammation.

Methods: Targeting was evaluated by flow cytometry and confocal microscopy. NGs' anti-inflammatory properties were studied in vitro on TNF-stimulated and non-stimulated hACs and, ex vivo, on cartilage explants. qPCR and ELISA of various markers assessed anti-inflammatory effect. Smooth muscle cell (SMC)-NGs were used as a non-MSC control.

Results: Flow cytometry showed that NGs can target hACs' 2 times more efficiently compared to SMC-NGs. Moreover, NGs showed 4 times higher targeting to TNF-stimulated hACs. Targeting was confirmed by confocal microscopy and imaging flow cytometry which showed that NGs bound the membrane and were taken up by the cells. Similar results were achieved in human explants where the particles showed 4 times higher binding to TNF-stimulated explants. To test the anti-inflammatory effect, different markers such as NO2, IL6, PGE2 and MMP13 were analysed. Our data showed that NGs reduce inflammation by more than 50% both at the protein and RNA level.

Summary/Conclusion: Here we provide a proof-of-concept for the utility of NGs with intrinsic capabilities for targeted cartilage regeneration, either as a standalone biological or as a carrier for the targeted delivery of therapeutics, such as anti-inflammatory agents and growth factors. Ongoing in vivo studies are focusing on confirming the NGs' targeting and anti-inflammatory capacity.

Funding: This project has received funding from the European Union's Horizon 2020 research and innovation programme under Marie Sklodowska-Curie grant agreement No 642414

OF20.02

Combining virus-based therapeutics and EV therapy for the treatment of pancreatic cancer

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Introduction: Pancreatic cancer (PC) is a highly aggressive disease with unmet therapeutic needs. Recent advances in the use of cancer killing oncolytic viruses (OVs) as cancer therapeutic agents bring new hope to fight the notorious disease that is PC. Although OVs have shown promising results in certain cancers, some tumours remain resistant to OV therapy due to their inherent residual antiviral mechanisms. We hypothesized that the use of OV-encoded artificial microRNAs (amiRs) could help target the cellular antiviral components associated with the observed OV resistance and could also sensitize neighbouring tumour cells to OV therapy and small molecule inhibitors through the secretion of amiR-containing extracellular vesicles (EVs) from infected cells.

Methods: To find such amiRs, we passaged a viral library encoding ~16,000 unique amiRs in several PC cell lines and patient-derived xenograft samples to enrich for sequences that could enhance OV replication.

Results: We identified an amiR that improves PC cell killing (amiR-PC) when expressed from an OV. Target identification of amiR-PC revealed ARID1A as a key player in resistance to OV therapy in PCs. This target is of particular interest since its down-regulation acts in a synthetic lethal fashion with inhibition of the EZH2 methyltransferase. Combining an

amiR-PC-expressing OV with a small molecule inhibitor of EZH2 enhances PC cell death. Moreover, we have shown that amiR-PC is packaged in cancer cell-secreted EVs which have the ability to reach neighbouring naïve cells to sensitize them to EZH2 inhibition-mediated cell death and to spread the OV-mediated tumour killing effect throughout the tumour. These results translate into an impressive improvement in tumour debulking and survival in animal models of highly aggressive PC.

Summary/Conclusion: This work not only broadens our knowledge on the resistance of select tumours to oncolytic virotherapy and the EV-mediated bystander killing effect in OV-infected tumours, but it also provides new hope for a cure to the grim disease that is PC.

OF20.03

CD47, a "don't eat me signal" expression in ovarian cancer cells were regulated by circulating exosomes – the therapeutic potential of targeting exosomes by inhibiting immune evasion

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Introduction: CD47, a "don't eat me" signal, is overexpressed on the surface of various tumours to allows tumour immune evasion. However, the role and regulation of CD47 in high grade serous ovarian carcinoma (HGSOC) remains undetermined. CD47 is known to be present on exosomes. Herein, we tested the following hypothesis that CD47 present on exosomes mediates immune evasion of cancer cells from macrophages.

Methods: Prognostic significances of CD47 expression in HGSOCs were examined using a public database including 1656 HGSOC patients (Kaplan-Meier Plotter; http://kmplot.com/analysis) and validated with 80 HGSCOs treated at Osaka University Hospital between 2013 and 2017. CD47 expression in exosomes derived from several HGSOC cell lines were examined by western blot. The effect of exosomal CD47 in HGSOCs was examined by inhibiting exosome secretion with GW4869, or by inhibiting exosome uptake with E1PA. Further, the co-culture experiments of HGSOCs with THP-1-derived macrophage were performed and the effect of exosomal CD47 on phagocytosis was analysed.

Results: High CD-47 expression was correlated with poor prognoses of HGSOC patients compared with low CD-47 expression (19.0 months vs. 23.6 months in overall survival (OS)). Exosomes derived from SKOV3ip1, OVCAR3 and A2780 cell lines showed strong CD47 expression. The inhibition of exosome secretion and uptake by GW4869 and E1PA inhibited CD47 expression in ovarian cancer cells, suggesting that CD47 is released from cells via exosomes and thereafter recycled via pinocytosis. The co-culture assay revealed that the inhibition of exosomal CD47 enhanced the phagocytosis of macrophage-like cells against cancer cells, which may lead to cancer cell survival *in vivo*.

Summary/Conclusion: CD47 expression was correlated with poor OS in HGSOC patients, suggesting the importance of immune evasion. CD47 was expressed on exosomes and the inhibition of exosome recycling enhanced the phagocytosis of macrophage-like cells against cancer cell through the down-regulation of CD47 expression in cancer cells. Our data indicates that cancer derived exosomes can be considered as a therapeutic target of HGSOCs.

OF20.04

The impact of *in vitro* ageing on the release of extracellular vesicles from human mesenchymal stem cells

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Introduction: Ageing increases the risk of bone degenerative diseases, which are partially caused by ageing-related changes in mesenchymal stem cells (MSCs). Both *in vitro* ageing (reflected by the passage number in culture) and ageing (donor age) reflect a loss of regenerative capacity in terms of the proliferation and osteogenic differentiation of MSCs. Extracellular vesicles (EVs) secreted from MSCs have been shown to exert therapeutic effects that contribute to the regeneration of various tissues, but there is scarce information on whether ageing, particularly *in vitro* ageing, influences the release of EVs by MSCs.

Methods: An *in vitro* ageing model in which low- and high-passage cells (LP and HP MSCs, respectively) represent "young" and "aged" cells, respectively, was utilized. Both LP and HP EVs were characterized by NTA and WB. The EV protein contents were further explored and the functions of LP and HP EVs on the survival and proliferation of MSCs were investigated.

Results: The results showed that *in vitro* ageing retained the phenotypic signature of MSCs but resulted in morphological changes and decreases in the proliferation and osteogenic differentiation capacity of the cells. Both LP and HP MSCs secreted EVs with similar characteristics in terms of size and typical exosomal

protein markers, but HP MSCs secreted more EVs than LP MSCs. The global proteome of the LP and HP EVs revealed that the vast majority of the identified proteins were in fact associated with EVs. The most abundant proteins in LP and HP EVs shared similar but not identical functional characteristics, and the proteins showing significant differential expression between HP and LP EVs were predicted to be enriched in Gene Ontology biological process terms mainly related to transport and secretion and to pathways regulating cellular morphology, growth/proliferation and development. Both LP and HP EVs promoted MSC survival and proliferation in autocrine and paracrine manners, and the degree of proliferation was dependent on the applied EV dose and associated with the characteristics of the recipient cells.
Summary/conclusion: The above-described results demonstrate that *in vitro* ageing influences the secretion of EVs by MSCs, particularly the number and protein cargoes of the EVs.

OF20.05

Novel role of BCR-ABL-containing leukemic extracellular vesicles in controlling the function of regulatory T cells

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Introduction: BCR-ABL-positive chronic myeloid leukemia (CML) has only recently been recognized as a malignancy associated with an immunosuppressive microenvironment, which includes increased amount of Foxp3+ regulatory T cells (Treg). However, mechanisms driving Treg differentiation and function in CML are mostly unknown. We hypothesize that extracellular vesicles (EVs) released by leukemic cells may be engaged in this phenomenon and mediate immunosuppression both inside and outside the CML bone marrow niche.
Methods: We isolated EVs from murine BCR-ABL-expressing progenitor cells, using differential centrifugation. EVs were characterized using TEM, Western blotting, Nanosight analysis and uptake of EVs by lymphocytes was analysed by flow cytometry. Influence of CML-derived EVs on Treg was analysed in different *ex vivo* settings, such as an *in vitro* suppression assay and differentiation of Treg on dendritic cells. To precisely analyse Foxp3 expression in Treg, we have used cells from B6.Cg-Foxp3tm2Tch mice that co-express Foxp3 and EGFP. Treg in blood of CML patients were analysed using 13-colour flow cytometry.
Results: Leukemic EVs potentiate suppressive function of regulatory T cells. This effect is driven by EV-mediated upregulation of Foxp3 – a transcription factor responsible for Treg suppressive phenotype. Proteomic analyses revealed that CML-derived EVs contain BCR-ABL oncoprotein. Interestingly, further functional studies revealed that inhibition of kinase activity of BCR-ABL in EVs has abolished the increase in Foxp3 level in EVs-treated Treg. Similarly to our *in vitro* findings, also Treg in CML patients seem to have more suppressive phenotype, as demonstrated by e.g. larger amount of highly suppressive CD39+ Tregs.
Summary/conclusion: CML-derived EVs seem to modulate immunosuppression in leukemia, by increasing suppressive activity of regulatory T cells. This effect is largely driven by BCR-ABL contained in leukemic EVs. However, precise mechanism of this regulatory pathway is yet to be dissected.
Funding: Grants from National Science Centre: 2013/10/E/NZ3/00673 to KP, 2018/29/N/NZ3/01754 to JS and Foundation for Polish Science grant TEAM TECH Core Facility Plus/2017-2/2 to KP.

OF20.06

Immunomodulatory function of human mesenchymal stromal cells (MSC)-derived extracellular vesicles (EVs) on type-i interferon response in human plasmacytoid dendritic cells (pDCs) and its therapeutic effect on murine lupus model

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Introduction: Immunoregulatory effect of mesenchymal stem cell (MSC) is attributed to extracellular vesicles (EVs) secretion. Given its effectiveness in preclinical studies of autoimmune disease, no one has examined its effect on SLE pathogenesis, signify by excessive type-I IFN production by plasmacytoid dendritic cells (pDCs) and animal models.
Objective: To investigate the effect of MSC and MSC-EVs on regulating cytokines production in pDCs, and the immunomodulatory role of MSC-EV in NZB/W F1.
Methods: htMSC (immortalized human MSCs), was cultured in CDPF medium for 48 h. EV were isolated by ultracentrifugation at 100,000*g*, 3 h, 4°C and characterized. Comparison of immunosuppressive function between htMSC-EV and TSG-6 knockdown htMSC on TLR9-mediated cytokine production in pDC was determined with GEN2.2, a human pDC cell-line, following

activation by CpG-A, and analysis by qPCR and ELISA. Finally, we compared the survival, pDC IFN-α intracellular expression and serum IgG autoantibodies expression of htMSC-EV-treated NZB W/F1 mice with those that received htMSC cellular treatment, and PBS control-group.

Results: Upon activation of TLR9 by CpG-A, IL-1ß, TNF-α and IFN-α transcription was upregulated in GEN2.2. Such response was reduced when CpG-A-primed GEN2.2 were co-cultured with htMSC. Knockdown of TSG-6 in htMSC dampened its capacity to suppress IL-1ß, TNF-α, IFN-α and IRF7 transcription in GEN2.2. To find out whether MSC exert its immunosuppressive effect by means of EV, we isolated EVs from hTERT MSCs and found htMSC-EV contained TSG-6 protein. Co-culture of htMSC-EV with CpG-A-primed GEN2.2 resulted in downregulation of IFN-α transcription and protein expression, mediated via reduction in total and phospho-IRF7. NZB/W F1 mice treated with htMSC-EV resulted in improved survival, followed by reduction in splenic pDC IFN-α expression, and IgG autoantibodies in serum.

Summary/conclusion: We showed that MSC downregulated TLR9 activation in human pDCs, in a TSG-6 dependent manner. Furthermore, htMSC-EV contains TSG-6 and suppress IFN-α response in CpG-A-activated pDCs through reducing total and phospho-IRF7. Finally, htMSC-EV treatment improved survival, and modulated pDCs and serum IgG autoantibodies expression in NZB/W F1 mice.

Symposium Session 21: EVs in Migration and Cancer
Chairs: Akiko Takahashi; Yoshinobu Takakura
Location: Level 3, Hall B

16:30–18:00

OF21.01

Targeting Rab27a in pancreatic cancer

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers with very limited therapeutic options. PDAC lesions are unique due to their extensive desmoplastic reaction and sparse cancer cells, highlighting the potential role of cell communication in PDAC progression. Despite cell communication being intrinsically involved in tumour progression, this process of tumorigenesis is still off the cancer therapy landscape. Exosomes have emerged as crucial mediators of intercellular communication in cancer. Rab27a and –27b have been described as key players in cancer exosomes release.
Methods: Therefore we decided to evaluate if inhibition of cancer exosomes communication could represent a novel therapeutic strategy in PDAC.
Results: We demonstrate that Rab27a, but not Rab27b, expression correlates with poor survival in patients with metastatic PDAC, but the same is not true for early stage PDAC. We further demonstrate that Rab27a knockout in pancreatic cancer cells is lethal, further stressing the crucial role of Rab27a for cancer cells survival. When using an inducible TetON knockdown system for Rab27a, downregulation of this protein impairs tumour growth in orthotopic models and, most strikingly, inhibits liver metastatic colonization. Next we evaluated Rab27a, –27b, –5 and –7 expression during disease progression in a genetically engineered mouse model (GEMM) that spontaneously develops PDAC (KPC) and reflects the human disease. Rabs expression is dynamic during the different stages of disease progression, but only Rab27a shows an increased expression in metastatic lesions. Using a Rab27a small molecule inhibitor in KPC mice we see a decrease in the number of liver macro-metastasis and increase in overall survival. In addition, we developed a conditional and inducible Rab27aKO mouse and show that pancreas conditional KO of Rab27a does not affect the normal development and physiology of the pancreas.
Summary/Conclusion: We are currently assessing the effects of Rab27a conditional KO in PDAC GEMMs.
Funding: Project NORTE-01–0145-FEDER-000029 from NORTE 2020. IF/00543/2013/CP1184/CT0004, PTDC/BIM-ONC/2754/201 and, POCI01-0145-FEDER-32189 from FCT – Foundation for Science and Technology. FAZ Ciencia Award from Astrazeneca Foundation.

OF21.02

Roles of lysyl oxidase like 2 (LOXL2) in exosomal fraction on lymph node metastasis of head and neck squamous cell carcinoma

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Introduction: The secretory enzyme lysyl oxidase like 2 (LOXL2) is assumed to contribute to tumour progression through participation in cellular events including remodelling extracellular matrix and epithelial-mesenchymal transition. In a previous study, we identified elevated expression of LOXL2 in human head and neck squamous cell carcinoma (HNSCC) lymph node metastases, and localization of LOXL2 in exosomal fraction of a metastatic HNSCC cell. Here we assessed the significance of LOXL2 in the metastasis and of liquid biopsies for detecting HNSCCs and their risk of the metastasis.
Methods: A mouse model of lymph node metastasis of HNSCC was established to assess metastatic activity and responsible factors for the metastasis. LOXL2 protein expression as well as lysyl oxidase enzyme activity were assessed in exosomal fraction of human metastatic HNSCC cell. Effects of LOXL2 knockdown on the metastasis was assessed in the mouse model.

LOXL2 levels were assessed in immunohistochemistry and immunoblotting in HNSCC tissues as well as serum from patients. Exosomal LOXL2 levels from further 38 serum samples from HNSCC patients were subjected to statistical analysis.

Results: LOXL2 localization was identified in exosome from metastatic HNSCC cell but not in non-metastatic cancer cell. Knockdown of LOXL2 attenuated the lymph node metastasis. Immunoblot analyses revealed that LOXL2 was present in human serum exosomal fractions, and the mean LOXL2 level was approximately three-fold higher in patients than healthy volunteers. Immunohistochemical LOXL2 staining was detected in HNSCC cells in a human tongue HNSCC sample. Further measurements of exosomal LOXL2 showed higher LOXL2 levels in patients.

Summary/Conclusion: Elevated serum exosomal LOXL2 levels can be an indicator of HNSCC. A follow-up clinical study will be required to determine the clinical utility of using LOXL2 to diagnose HNSCC and/or determine the risk of metastasis.

Funding: This study was supported in part by grants from Ehime University (#0101010010, #0101050003, and #0101390003), Daiwa Securities Health Care Foundation (#14–23), and the Ministry of Education, Culture, Sports, Science, and Technology of Japan (15K10809 and 18K09379).

OF21.03

Growing old disgracefully – a novel role of extracellular vesicles in bone invasion

Areeg A. Elmusrati, Daniel Lambert and Syed Ali Khurram

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Introduction: Bone invasion is a common feature of oral squamous cell carcinoma (OSCC) and is associated with poor prognosis. The mechanism of OSCC bone invasion remains unclear, but our recent work indicated a key role for cancer-associated fibroblasts (CAF)

Methods: In this study we sought to investigate whether senescent fibroblasts and derived extracellular vesicles (EV) play a role in bone invasion in OSCC. Immunohistochemistry (IHC) for senescence markers p16INK4a and dipeptidyl peptidase 4 (DPP4) was carried out on bone resection cases with cortical and medullary OSCC invasion. Senescence in normal oral fibroblasts (NOF) was experimentally induced through replicative mitotic exhaustion, as well as exposure of NOF at low passage to hydrogen peroxide, and the chemotherapeutic drug cisplatin. Senescence-associated beta-galactosidase (SA-βgal) activity was monitored to confirm senescence induction. Receptor activator of nuclear factor kappa-B ligand (RANKL), a key pro-osteoclastogenic factor, and p16INK4a transcript abundance was assessed by qPCR. Conditioned media was collected and an enzyme-linked immunosorbent assay used to detect soluble RANKL protein secretion. Furthermore, osteoclastogenesis and pit formation on a synthetic bone substrate was assessed in response to proliferating and senescent NOF-derived EV conditioned media.

Results: Readily detectable p16INK4a and DPP4 expression was observed in fibroblastic stroma adjacent to bone, indicative of the presence of senescent fibroblasts. A significant increase in RANKL and p16INK4a expression was observed following 15 days of senescence induction. RANKL secretion by NOFs was significantly upregulated following *in vitro* induction of senescence. Conditioned media from senescent oral fibroblasts provoked a marked increase in osteoclastogenesis, shown by an increase in tartrate resistant acid phosphatase activity, multinucleation and osteoclastic resorption/pit formation.

Summary/Conclusion: Here we provide novel evidence that senescent oral fibroblasts and derived EVs play an active role in OSCC bone invasion. The ability of senescent fibroblast-derived factors to promote osteoclastogenesis may have implications more broadly for age-related bone pathologies, and this is the focus of our ongoing investigations.

OF21.04

The multifaceted role of breast cancer-derived extracellular vesicles in brain metastasis

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Introduction: Breast cancer brain metastasis is often associated with a dismal prognosis. Elucidation of the early events that lead to brain metastasis will pave the way to identifying potential diagnostic and therapeutic targets for early intervention. We have previously shown that extracellular vesicles (EVs) derived from the brain-seeking MDA-MB-231 breast cancer cell line can increase brain metastasis growth. To investigate the mechanisms underlying the EV-induced facilitation of brain metastasis, we studied the mechanisms with which EVs interact with and modulate the blood brain barrier (BBB), as an initial niche for tumour cell growth.

Methods: EVs were isolated from the parental MDA-MB-231 breast cancer cell line (P-EVs) and its brain-seeking variant (Br-EVs). Through retro-orbital and intracardiac injection of EVs in mouse and zebrafish models, we studied the distribution of EVs to the brain. A combination of *in vitro* and *in vivo* BBB models was used to study the mechanisms with which EVs interact with an intact BBB. We next conducted continuous *in vitro* and *in vivo* treatment with EVs to elucidate the effects of EVs on the behaviour of the luminal and abluminal components of the BBB.

Results: Our distribution studies demonstrated that breast cancer-derived EVs could enter the brain parenchyma via an intact BBB. Using state-of-the-art models of the BBB and high-resolution microscopy, we have identified, for the first time, the mechanisms with which Br-Ex interact with the endocytic pathway in brain endothelial cells to cross the endothelium. Interestingly, our mechanistic studies showed that through transferring miRNAs, Br-EVs could modulate the endothelial endocytic pathway to decrease EV degradation. Moreover, we have shown that following their transport across the brain endothelium, Br-EVs can exclusively alter the expression profile of astrocytes to provide a suitable environment for metastatic growth.

Summary/Conclusion: These findings indicate that EVs derived from a brain-seeking subpopulation of breast cancer cells can exclusively modify the physiological regulation of the BBB at multiple levels to accelerate metastatic growth in the brain microenvironment.

Funding: This work was supported by the Breast Cancer Research Foundation and NIH R01CA185530.

OF21.05

Exposed aminophospholipids enriched in a subtype of small extracellular vesicles from tumour cell lines

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Abstract: Aminophospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) normally exist in the inner leaflet of the plasma membrane. Tumour cells, however, expose PS on their surfaces and release the extracellular vesicles (EVs) enriched with the exposed PS, which have been proposed to play an important role in communication between tumour cells and other surrounding or distal cells. We have recently identified a subtype of small EVs (sEVs) from tumour cell lines that were enriched with exposed PS; this subtype has lower density, larger size, more negative zeta potentials and lower abundance of exosomal proteins. Because PS and PE have often been reported to change their membrane localization in a closely associated manner, in this study, we aimed to examine if PE is also present in this subtype of sEVs.

Methods: An sEV fraction was prepared from a conditioned medium of tumour cell lines (HT-29 and HT-1080) that were propagated in a serum-free medium for approximately 68 h. After differential centrifugations (10,000*g* for 30 min and 160,000*g* for 70 min) and filtration with a 0.22-μm pore filter, the sEVs were further differentiated by continuous density-gradient centrifugation (8–40% iodixanol, 100,000*g*, 17 h) into 10 fractions. Thereafter, the fractions were washed with phosphate-buffered saline and analysed by Western blotting, silver staining, nanoparticle tracking and atomic force microscopy (AFM). To detect PS or PE on the surfaces of the vesicles, sEVs were labelled with gold nanoparticles (GNPs) using MFG-E8 or duramycin, respectively, followed by AFM observation.

Results: Continuous density-gradient centrifugation showed two subtypes of sEVs. One subtype was enriched with canonical exosome markers, including CD63, CD81, Alix and Tsg101, and had a density of 1.10 g/ml. The other subtype, however, was scarce for these markers and had a lower density of 1.04 g/ml. The estimation of the amounts of exposed PS and PE by GNP in AFM showed that the second subtype of sEVs had exposed PE as well as PS.

Summary/conclusion: The subtype of sEVs with lower density and fewer canonical exosome markers in density-gradient centrifugation contained not only exposed PS but also PE, which defined a new subtype of sEVs from tumour cells.

Funding: This work was supported by JSPS KAKENHI Grant Numbers 16K07152 to SM and 17H06255 to KS.

OF21.06

Mesenchymal stem cells-derived exosomes present natural migration and homing abilities to specific neuropathological areas

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Israel, Sacklar School of Medicine, Department of Human Genetics and Biochemistry Tel Aviv University, Israel, Tel Aviv, USA

Introduction: Though exosoemes have been found to cross the blood–brain barrier, their migration and homing abilities within the brain remain unstudied. We have recently developed a method for longitudinal and quantitative *in vivo* neuroimaging of exosomes, based on the superior visualization abilities of CT, combined with gold nanoparticles as labelling agents. Here, we used this technique to track the migration and homing patterns of intranasally administrated exosomes derived from bone marrow mesenchymal stem cells (MSC-exo) in different brain pathologies, including stroke, autism, Parkinson's disease and Alzheimer's disease. We found that MSC-exo specifically targeted and accumulated in pathologically-relevant murine models brains regions up to 96 h post administration, while in healthy controls they evacuated. The neuro-inflammatory signal in pathological brains was highly correlated with MSC-exo accumulation. In addition, MSC-exo were selectively uptaken by neuronal cells in the pathological regions.

Methods: Exosomes were extracted from human bone marrow mesenchymal stem cells. They were loaded with glucose-conjugated gold nanoparticles and were given via intranasal administration to mice with different pathologies. All mice were scanned with CT 1, 24 and 96 h post administration. Moreover, using PKH26 MSC-exo were labelled and were visualized with whole brain florescence.

Results: Altogether, our DATA suggests that MSC-exo present distinct neurodistribution which is pathology-specific in each of the mice models visualized both *in vivo* and ex-vivo.

In both the induced stroke and Parkinson's models, the MSC-exo were visualized mainly in the damaged tissue (Striatum). In Alzheimer's model, they were visualized mainly in the hippocampus, and in the Autism mice model, they were visualized both in the prefrontal cortex and the cerebellum. Interestingly, in healthy mice the exosomes did not home to any specific location and the signal was lost 24 h post administration both *in vivo* and *ex vivo*. In the damaged tissue, the MSC-exo were found mainly in the neurons and not in other cells.

Summary/conclusion: Taken together, these findings can significantly promote the application of exosomes for therapy and targeted drug delivery in various brain pathologies via intranasal administration.

Symposium Session 22: Novel Methods of EV Analysis
Chairs: An Hendrix; John Nolan
Location: Level B1, Hall A

16:30–18:00

OF22.01

Biolayer interferometry – extracellular vesicles (BLIEV) platform for liquid biopsy of ovarian cancer

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Introduction: Rigorous efforts are being addressed to tackle the hurdles in extracellular vesicle (EV)-based cancer diagnostics – in particular liquid biopsies. We introduce a promising optical platform, biolayer interferometry – EV (BLIEV) for EV-based liquid biopsies. BLIEV utilizes "dip-and-read" optical fibre biosensors without any microfluidics that can be clogged by crude and complex sample matrices.
Methods: A ForteBio Octet RED384 System and SSA Streptavidin sensor tips were used for the BLI. EVs were collected from the serum of 16 ovarian cancer (OvCan) patients and 16 healthy (H) individuals. A group of 16 test samples was also prepared by spiking-in several concentrations of OvCan EVs into healthy human plasma. The sensor tips were functionalized with biotinylated CD9 or CD63. Bulk EVs from OvCan and H preparations were captured by the BLIEV-CD9 or BLIEV-CD63 biosensors. The same procedure was repeated for the spiked-in clinical test samples. Cancer-specific signals were acquired by dipping the EV-containing BLIEV biosensors to the solution of an in-house cyclic reporter peptide, LXY30, which has been demonstrated to bind the α3β1 integrin overexpressed on OvCan cells and EVs.
Results: The EV capturing efficiency of BLIEV-CD9 and BLIEV-CD63 sensors was very high, enabling the subsequent detection with LXY30. Regardless the used biosensor type, high signals were recorded in the bulk-capturing phase for OvCan EV preparations while the healthy EV samples gave negligible signals. Titration of the prepared spiked-in clinical test samples yielded remarkably high OvCan detection specificity (>90%), and the sensitivity reaching as low as < 1500 EVs/μL. Our integrin-binding reporter peptide LXY30 enabled significant detection specificity towards the OvCan EVs.
Summary/Conclusion: We have developed a robust, sensitive, label-free, cost-effective, and high-throughput BLIEV platform for EV-based liquid biopsies. Up to 16 clinical EV samples can be analysed in parallel and total 384 samples in one run. Processing 16 EV samples required less than 2 min. To our knowledge, this is the first time BLI has been used as a diagnostic tool. We strongly believe that the remarkable sensitivity and specificity of our BLIEV platform exhibit clinical significance for the next-generation cancer diagnostics.
Funding: Sigrid Juselius Foundation, Finland.

OF22.02

Enhanced detection and visualization of exosomes with interferometric reflectance imaging

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Introduction: Biological sensors that detect whole viruses and exosomes with high specificity, yet without chemical labelling, are promising because they generally reduce the amount and complexity of sample preparation required by molecular amplification methods and may improve measurement quality by retaining information about nanoscale biological structure. We developed an optical sensing technology, Interferometric Reflectance Imaging Sensor (IRIS), a multifunctional platform for quantitative, label-free and dynamic detection. In high-magnification modality, Single-Particle Interferometric Reflactance Imaging (SPIRI) has the ability to detect and characterize individual biological nanoparticles. We have recently improved the contrast and spatial resolution of SPIRI by pupil function engineering and computational imaging.
Methods: In SPIRI, the interference of light reflected from the sensor surface is modified by the presence of particles producing a distinct signal that reveals the size of the particle that is not otherwise visible under a conventional microscope. Using this instrument platform, we have demonstrated label-free identification and visualization of various viruses in multiplexed format in complex samples in a disposable cartridge. Recently, our technology was applied to detection of exosomes and commercialized by Nanoview Biosciences for quantified measurement of exosomes on dry sensor chips. We are currently focusing on

various in-liquid detection as well as further improvement of the technique using pupil function engineering.
Results: By acquiring multiple images with a partitioned pupil (resulting in structured illumination) and computational imaging, we have demonstrated significant improvement in visibility of low-index nanoparticles in liquid. Furthermore, spatial resolution has been improved beyond the diffraction limit approaching 100 nm in the visible microscopy. We have developed compact and inexpensive sensor chips and microfluidic cartridges allowing for study of biological particles (exosomes and other extracellular vesicles) directly in the bodily fluids without labels.
Summary/Conclusion: In summary, we have demonstrated improved visibility of exosomes in SPIRI using pupil function engineering.
Funding: EU-INDEX

OF22.03

Proximity assays for detection and characterization of exosomes

Masood Kamali-Moghaddam, Ehsan Manouchehri, Alireza Azimi, Qiujin Shen, Radiosa Gallini and Claudia Fredolini

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Introduction: Exosomes receive an increased attention in basic biology as well as in medicine. They are shown to be involved in many biological processes, and are proven to hold great potentials as diagnostic and therapeutic tools. However, there is an unmet need for new and improved technologies for quantitative and qualitative characterization of exosomes to meet challenges related to these vesicles, such as low concentrations in body fluids, the small size of the exosomes or the low copy numbers of antigens present on the surface of the exosomes.
Methods: We have developed a large number of affinity-based proximity assays for single- and multiplex detection of proteins and large complexes with high specificity and sensitivity. Several of these technologies, such as proximity ligation assay combined with flow cytometry readout, multiplex proximity extension assays and proximity barcoding assays, are used for sensitive detection and characterization of individual exosomes.
Results: Commonly, in these assays the exosomes are recognized by several affinity binders, each equipped with a DNA oligonucleotide. Upon binding of the target exosomes by the affinity probes, the DNA oligonucleotides are brought in proximity, subjected to enzymatic ligation or polymerization, which results in formation of an amplifiable reporting molecule. The use of multiple recognition events in combination with signal amplification allows detection of exosomes with high specificity and sensitivity.
Summary/Conclusion: Here, we discuss the application of proximity assays for sensitive detection of exosomes in body fluids, to visualize the uptake of exosomes by cells, and the potential of such approach to be used to better understand the biology of the exosomes and to identify exosomes as disease biomarkers.

OF22.04

A 96 well plate format lipid quantification assay with improved sensitivity for standardization of experiments with extracellular vesicles

Tamas Visnovitz[a], Xabier Osteikoetxea[b], Barbara W. Sódar[c], Judith Mihaly[d], Péter Lőrincz[e], Krisztina V. Vukman[a], Eszter Ágnes Tóth[a], Anna Koncz[a], Inna Székács[f], Robert Horváth[f], Zoltan Varga[g] and Edit I Buzás[c]

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Introduction: Current EV studies usually standardize EV samples on the basis of their protein content, particle number or both. Even with this latter approach may lead to inaccuracy and overestimation of the EV concentration. Lipid bilayers are defining components of EVs. Therefore, a lipid-based quantification, especially in combination with protein content and/or particle count determination, appears to be a straightforward approach for quantification of EVs. Here we set the goal to improve the sensitivity of the previously reported sulfo-phospho-vanillin (SPV) lipid assay.
Methods: We to replace the traditional purified lipid standards (diluted in organic solvents) with an aqueous phase liposome standard (DOPC), and we optimized the concentration of the vanillin reagent of the assay. Results of the lipid assay were compared with the previously described ATR-FTIR spectroscopy-based lipid quantification approach. The assay was validated with EPIC biosensor system, qNano, commercially available lipid assay and commercial LDL. Using the optimized lipid assay, we tested liposomes of known composition as well as EVs secreted by four different cell lines. EV markers were documented by immune electron microscopy.
Results: Elimination of organic solvents from the reaction mixture abolished the background colour that previously interfered with the assay. Comparison of

the optimized assay with a commercial lipid kit (also based on the original SPV lipid assay) showed an increase of sensitivity by approximately one order of magnitude, and the lipid-based quantification of EV samples have clearly increased the reliability of the experiments.

Summary/Conclusion: The optimized lipid assay with improved sensitivity provides a fast, reliable and sensitive test that addresses an existing need in EV standardization. This optimized lipid assay for EV lipid measurements can be as easy as a simple BCA test for protein determination.

Funding: NVKP_16-1-2016-0017, OTKA11958, OTKA120237, OTKA PD112085, VEKOP-2.3.2-16-2016-00002 and VEKOP-2.3.3-15-2016-00016, KH_17 grant, ERC hu and Lendület, Institutional Higher Education Excellence Program of the Ministry of Human Resources in the theme "Therapeutic development". János Bolyai Research Fellowship of HAS.

OF22.05

Characterization of exosomes-based on their unique dielectric properties by a novel electrical impedance measurement system

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Introduction: Exosomes are composed of a lipid bilayer membrane containing nucleic acids, proteins and lipids in the lumen and their compositions reflect their cell of origin. Thus, when the secreting cells are in abnormal microenvironments, the exosomes undergo the compositional changes. We have developed a new class of electrical impedance measurement system to non-invasively characterize exosomes based on their unique dielectric properties. Although, the biophysical properties of exosomes such as size, density and shape have been characterized before, their dielectric properties have not been investigated.

Methods: An electrokinetic-based system has been developed to characterize the dielectric properties of exosomes extracted from human hepatocellular carcinoma (HuH-7) cells under different culture conditions. Extracted exosomes were initially trapped with dielectrophoresis and further characterized by their dielectric properties as 0.2Vpp was swept from 1 kHz to 50 MHz.

Results: The principle of the impedance measurement was adapted from the Maxwell's mixing theory applied to analyse the dielectric behaviour of cells. Opacity was defined as the ratio of impedance magnitude at high frequency (>1 MHz) to the low frequency (e.g. 500 kHz), which provided a parameter independent of the number of vesicles, reflecting the changes in dielectric properties including their membrane capacitance and cytosolic conductance. Extracted exosomes from different cell of origins were measured five times and the result showed the changes in opacity measurements at the intermediate and high frequency ranges which represents the difference between the membrane composition and cytosolic conductance of the exosomes.

Summary/Conclusion: A new class of electrical impedance measurement system was developed with the capability to characterize and distinguish exosomes based on their unique dielectric properties as their biogenesis was subjected to systematic changes under different culture conditions. This technique can be further utilized for classification of exosomes based on their cell of origin and can be evolved as a diagnostic tool for characterizing the pathogenic exosomes.

Funding: UC Faculty Development Fund.

OF22.06

A snorkel-tag based method for *in vivo* isolation of recombinant extracellular vesicles

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Introduction: Extracellular vesicles (EVs) emerged as an important mode of cell-to-cell communication in both normal and pathological conditions by transferring the cargo from donor cell to recipient cell. It is their apparent natural ability to transfer cargo from donor cell to recipient cell and thus regulating via paracrine or endocrine mode. Over a decade, lot of research has been done to understand the omics, mode of secretion and uptake mechanisms. However, trafficking of EVs *in vivo* is still poorly understood.

Methods: We used recombinant tetraspanin (tetraspanin with C-terminus snorkel tag (1)) as a tool to understand trafficking of EVs *in vivo*. As a first step we established a method for isolating functional EVs carrying recombinant tetraspanins from stably expressing cells *in vitro*. The presence of snorkel-tagged

tetraspanins on EVs are not affecting the surface protein signature (2). This method uses a combination of anti-HA (hemagglutinin) affinity matrix and Prescission protease to isolate EVs from cell culture supernatants without damaging the integrity of the EV membrane.

Results: EVs isolated by this method are further characterized by using multiplex bead-based flow cytometry assay and electron microscopy. The multiplex bead-based assay results showed us that we are able to pull out EVs carrying only snorkel tag from a mixture of different EVs from different sources. Furthermore, we plan to spike in human recombinant EVs into mouse plasma and isolate recombinant EVs from this complex matrix using this method and confirm by multiplex bead-based assay. In addition, to determine the functionality of recombinant EVs, we used CRE-LoxP method (3) to confirm the recombinant EV uptake in recipient cells.

Summary/Conclusion: Ultimately, we are comparing the RNA content of recombinant EVs isolated by snorkel-tag to CD81+ affinity purified EVs with the total EV population in order to investigate the specific RNA loading by RNA seq.

Funding: This work supported by the FWF Doctoral Program BioToP [W1224]

Plenary Session 3: RNA
Saturday 27 April
Chairs: Jan Lötvall; Marca Wauben
Location: Level 3, Hall B

10:00–11:20

piRNA biogenesis and functions in *drosophila*

Mikiko C. SIOMI

University of Tokyo, Tokyo, Japan

PIWI-interacting RNAs (piRNAs) are small non-coding RNAs enriched in animal gonads where they arm race with transposons to maintain germline genome integrity. Although transposons are powerful agents contributing to evolution, they are also regarded as selfish DNA parasites. Indeed, loss of piRNAs causes derepression of transposons, leading to DNA damage and failure in gonadal development and fertility. Thus, piRNA-mediated transposon silencing is indispensable for animals that undergo obligate sexual production, including humans. Since the discovery of piRNAs, studies have intensively been conducted worldwide and fundamental features of the pathway have emerged. We now know that piRNAs are primarily produced from piRNA clusters, discrete intergenic elements composed of transposon remnants, and loaded onto PIWI proteins to form piRISCs. Cytoplasmic piRISCs silence transposons post-transcriptionally while piRISCs in the nucleus repress target genes co-transcriptionally. However, the molecular mechanism is not yet fully understood. To solve the problem, we have use *Drosophila* and *Bombyx* as model systems, particularly their cultured cell lines where piRNAs are fully functional in repressing transposons. The details of our new findings will be presented at the meeting.

EV as a novel therapeutic target for cancer metastasis

Takahiro Ochiya, Ph.D., Chief and professor

National Cancer Center, Tokyo and Tokyo Medical University

Extracellular vesicles, known as exosomes and microvesicles, serve as versatile intercellular communication tools. Increasing evidence has suggested that cancer cell-derived exosomes carry pathogenic components. Exosomal transfer of cancer pathogenic components enable long-distance-crosstalk between cancer cells and target organs and tissues, resulting in the promotion of the initial steps for pre-metastatic niche formation. Furthermore, the circulating exosome have also been of interest as a source for liquid biopsies. Circulating exosome in body fluids provides a reliable source of miRNAs, mRNAs, DNAs, proteins and oncometabolites for cancer biomarkers. We also suggest our current knowledge on the tumour-specific DNA methylome in exosomes effectively provide various messages on the physiological and pathological status of cancer patients. In this talk, we provide an overview of current research on exosomes in cancer. We also propose new therapeutic strategies by targeting cancer-specific exosomes to inhibit tumour metastasis.

Featured Abstracts- Session 2
Chairs:
Location: Level 3, Hall B

11:20–12:00

FA2.01

A novel CRISPR/Cas9-based reporter system enables detection of EV-mediated functional transfer of RNAs on a single-cell level

Olivier G. de Jong[a], Dan E. Murphy[b], Imre Mäger[c], Eduard Willms[c], Sander A.A. Kooijmans[b], Raymond Schiffelers[b], Samir El Andaloussi[d], Matthew J. A. Wood[c] and Pieter Vader[b]

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Introduction: In recent years, multiple studies have shown that extracellular vesicles (EVs) play a role in intercellular communication through transfer of RNAs. Unfortunately, our understanding of the mechanisms regulating EV-mediated RNA delivery and processing is lacking, due to the absence of suitable readout systems for functional RNA transfer. Here, we describe a novel highly-sensitive CRISPR/Cas9-based reporter system that, for the first time, allows direct functional study of EV-mediated transfer of small non-coding RNA molecules on a single-cell level.

Methods: We generated a CRISPR/Cas9-based stop-light reporter system, in which eGFP expression is activated upon functional delivery of targeting single-guide RNAs (T-sgRNAs). Donor cell lines were generated stably expressing either T-sgRNAs or non-targeting sgRNAs (NT-sgRNAs). Intercellular functional RNA transfer was assessed by measuring eGFP expression in reporter cells after direct co-culture, transwell co-culture, and upon addition of isolated EVs, using fluorescence microscopy and flow cytometry. The role of potential regulators of EV-mediated RNA transfer was assessed after RNAi-mediated target knockdown in reporter cells, prior to co-culture experiments.

Results: Expression of sgRNAs in donor cells and EVs was confirmed by RT-PCR. A significant activation of eGFP expression was observed in reporter cells after direct co-culture and transwell co-culture with donor cells expressing T-sgRNAs, but not NT-sgRNAs. Addition of EVs from cells expressing T-sgRNAs, and not NT-sgRNAs, also resulted in significant reporter activation. Reporter activation was substantially decreased after blocking EV production through addition of GW4869 or Rab27A knockdown in donor cells. Knockdown of multiple targets in endocytosis and/or intracellular membrane trafficking in reporter cells significantly decreased reporter activation, suggesting vital roles for these processes in EV-mediated RNA transfer.

Summary/Conclusion: Here we demonstrate a CRISPR/Cas9-based reporter system that for the first time allows the study of functional delivery of small non-coding RNAs with single-cell resolution. This novel approach allows the study of EV cargo processing in the context of functional RNA delivery, and may help to increase our understanding of the regulatory pathways that dictate the underlying processes.

FA2.02

From nanoscale to organisms: a multi-resolution imaging system of endogenously released extracellular vesicles with bioluminescence resonance energy transfer

Anthony Yan-Tang Wu[a], Yun-Chieh Sung[b], John Jun-Sheng Ko[c], Alan Ling Yang[d], Vanessa Guo[e], Yunching Chen[f] and Charles P. Lai[e]

[a]Department and Graduate Institute of Pharmacology, National Taiwan University, Taipei, Taiwan (Republic of China); [b]Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan (Republic of China); [c]Institute of Biomedical Engineering, National Tsing Hua University, Zuoying, Taiwan (Republic of China); [d]Institute of Atomic and Molecular Sciences, Taoyuan, Taiwan (Republic of China); [e]Institute of Atomic and Molecular Sciences, Academia Sinica, Taipei, Taiwan (Republic of China); [f]National Tsing-Hua University, Hsinchu, Taiwan (Republic of China)

Introduction: Accurate visualization and monitoring of EVs is paramount to the understanding of its biological mechanism. However, EV labelling with organic dyes such as DiI and PKH have an extended half-life (e.g. PKH26: >100 days) and self-assemble into micelles, which may lead to inaccurate interpretation of EV spatiotemporal properties. Furthermore, reporters which fuse EV and fluorescent proteins (e.g. CD63-GFP) may only detect specific EV subpopulations.

Methods: To overcome these limitations, here we developed a next-generation reporter to enable EV imaging from organismal to super-resolutions by engineering enhanced green fluorescent protein fused to a palmitoylation signal and nanoluciferase, termed PalmGp. HEK293T and HCA-1 cells were lentivirally transduced to stably expressed PalmGp as EV donor cells. *In vivo* EV and nanoscopic imaging were

achieved by *in vivo* imaging system (IVIS) and super-resolution radial fluctuations nanoscopy, respectively.
Results: PalmGp predominantly labels EV inner membrane, and exhibited a robust bioluminescent and BRET-GFP signals for EV imaging upon addition of its substrate, furimazine. IVIS imaging of liver cancer HCA1-PalmGp bearing mice showed sustained bioluminescent and fluorescent signals at the primary and metastatic sites, indicating the high sensitivity of PalmGp to enable biodistribution and clearance of endogenously released EVs. PalmGp-EVs could further be visualized at super-resolution (50–150 nm) to monitor EV subcellular trafficking.
Summary/Conclusion: To our knowledge, this is the first report of a multi-resolution reporter strategy that enables EV imaging. Efforts are currently underway in employing the PalmGp EV reporter to elucidate the spatiotemporal property and mechanism(s) of cancer EVs during disease progression.
Funding: Ministry of Science and Technology (MOST) grants104-2320-B-007–005-MY2 (C.P.L.), 106–2320-B-007–004-MY3 (C.P.L.), and Academia Sinica Innovative Materials and Analysis Technology Exploration (i-MATE) Program AS-iMATE-107–33 (C.P.L.)

Symposium Session 23: EV Engineering II
Chairs: Cherie Blenkiron; Thomas Kislinger
Location: Level 3, Hall B

13:00–14:00

OS23.01

exoTOPE: loading bioactive molecules into exosomes using a short-peptide fusion

Russell McConnell, Madeleine Youniss, Ke Xu, Kevin Dooley, Bryan Choi, Rane Harrison, Sonya Haupt, Damian Houde, Nuruddeen Lewis, Shelly Martin, Chang Ling Sia and Sriram Sathyanarayanan

Codiak BioSciences, Cambridge, USA

Introduction: Exosomes represent a promising therapeutic platform for the selective delivery of diverse classes of payloads; however, loading exosomes with non-native cargo molecules has historically been a significant barrier to unlocking this potential. We reasoned that it would be possible to load therapeutically relevant proteins into exosomes by identifying and co-opting peptide sequences that natively enrich proteins in exosomes.
Methods: Differential and density gradient ultracentrifugation were used to purify exosomes from cell culture supernatant. LC-MS/MS was used to identify proteins present in purified exosomes, the amino acid sequences of highly abundant proteins were analysed for common sequence features, and plasmids encoding candidate peptide sequences fused to cargo proteins were expressed in stably selected cells. The enrichment of fusion proteins in purified exosomes was assessed using biochemical, flow cytometric and functional analyses.
Results: Among the most abundant native exosomal proteins identified by LC/MS-MS were three members of the MARCKS family. All three MARCKS family members were found to strongly localize to purified exosomes when overexpressed as GFP fusions. Using truncated and point mutant versions of sequences derived from these proteins, we identified a seven amino acid consensus peptide sequence that is able to load non-native cargo proteins into the exosome lumen at extremely high levels, comprising up to ~10% of the total exosomal protein. Sequences containing this seven amino acid "exoTOPE" tag were used to load exosomes with cytosolic cargos such as fluorescent proteins, RNA-binding proteins and mRNA, Cas9, antigenic peptides and proteins, and the type 2 transmembrane protein CD40 ligand (CD40L). Exosomes carrying exoTOPE-CD40L activated antigen presenting cells in PBMC assays with similar EC50 values as free recombinant CD40L.
Summary/Conclusion: We have identified and refined a short peptide, exoTOPE, that can be used to load exosomes with diverse classes of cargos, including proteins and nucleic acids. The small size of this peptide tag makes this system readily adaptable to a wide variety of applications and represents a significant advance in our ability to engineer exosomes with biologically active cargos.
Funding: Funded by Codiak Biosciences.

OS23.02

Retrograde dicer-independent AGO-loading of extracellular single stranded miRNA in recipient human cells

Bartika Ghoshal[a] and Suvendra N. Bhattacharyya[b]

[a]CSIR_Indian Institute of Chemical Biology, Kolkata, India, Kolkata, India; [b]Molecular Genetics Division, CSIR-Indian Institute of Chemical Biology, Kolkata, India

Introduction: microRNAs are tiny regulator of gene expression that can be transferred between neighbouring cells in mammalian tissue to control the expression of genes in both donor and recipient cells. How the extracellular vesicle (EV)-derived miRNAs are getting internalized and become functional in target cells is an unresolved question.
Methods: We used mammalian cells in culture to study the EV-mediated miRNA delivery to target cells. Using miR-122 negative HeLa cells as recipient cells and miR-122 containing exososmes isolated from miR-122 positive cells, we have delineated the mechanistic detail of the import process.
Results: We have identified that, through a unique mechanism, the EV-associated miRNAs that are primarily single stranded can get loaded with the Ago proteins present in the target cells to become functional there. The loading of EV-derived miRNAs to host cells Ago proteins is not dependent on the Dicer1 that otherwise required for the loading of the Ago proteins with double stranded miRNAs before one strand get cleaved and dislodged from Ago2. The EV-derived miRNA loading of Ago2 happens on the endosomal membrane where the pH dependent fusion of the internalized EV membrane with endosomal membrane releases the miRNAs that

get loaded with unloaded Ago2 present on the endosomal membrane. This process is depenent on memebrane dynamics and restriction of memebrane dynamics either due to mitochondrial depolarization or other ways affects the loading of EV-derived miRNAs with Ago2. Leishmania donovani, a protozoan parasite affect membrane dynamics in infected macrophage cells and thus it restrict the internalization of miR-122 containing EVs that otherwise cause an inflammatory response in mammalian macrophage-a process detrimental for the pathogen.

Summary/Conclusion: therefore we conclude that Leishmania donovani Restricts Retrograde Dicer-Independent Loading of Extracellular Single Stranded miR-122 in Host Cell Agos to Prevent Inflammatory Response.

Funding: SERB, Dept of Science and Technology, Govt. of India and Swarnajayanti Fellowship Fund, Dept of Science and Technology, Govt. of India.

OS23.03

Engineering of extracellular vesicles for surface display of targeting ligands

Elisa Lázaro-Ibáñez[a], Anders Gunnarsson[b], Gwen O´Driscoll[b], Olga Shatnyeva[c], Xabier Osteikoetxea[d] and Niek Dekker[b]

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Introduction: Cell engineering is one of the most common strategies to modify extracellular vesicles (EVs) for therapeutic drug delivery. Engineering can be applied to optimize cell tropism, targeting, and cargo loading. In this study, we screened several EV proteins fused with EGFP to evaluate the surface display of the EV-associated cargo. In addition, we screened for EV proteins that could efficiently traffic cargo proteins into the lumen of EVs. We also developed a novel technology to quantify the number of EGFP molecules per vesicle using total internal reflection (TIRF) microscopy for single-molecule investigation.

Methods: Human Expi293F cells were transiently transfected with DNA constructs coding for EGFP fused to the N- or C-terminal of EV proteins (e.g., CD63, CD47, Syntenin-1, Lamp2b, Tspan14). 48 h after transfection, cells were analysed by flow cytometry and confocal microscopy for EGFP expression and EVs were isolated by differential centrifugation followed by separation using iodixanol density gradients. EVs were characterized by nanoparticle tracking analysis, western blotting, and transmission electron microscopy. Single-molecule TIRF microscopy was used to determine the protein number per vesicle at a single particle level, using monomeric EGFP as a reference.

Results: The screening of EGFP fused to the N- or C-terminal of EV proteins served as a quantitative method to identify protein candidates for the surface display of EV-associated cargo. Fusions to CD47 and luminal EV proteins with a snorkel domain allowed the display of EGFP at the surface of EVs, with CD47 as good candidate for surface display. Alternatively, fusions of EGFP to EV proteins with either C- or N-in topology like Tspan14 and CD63 allowed for loading of EGFP within the EV lumen. Single EV analysis using TIRF microscopy enabled the quantification of the average number of EGFP molecules per single engineered vesicle, which was between 15 and 136 EGFP/EV depending on the fusion protein.

Summary/Conclusion: The screening of EGFP-fusions to EV proteins revealed several protein candidates for both surface display and intra-luminal cargo loading in EVs. These results contribute to the understanding of EV biogenesis and are relevant for exploiting the potential of engineered EVs as drug delivery systems.

OS23.04

Endogenous drug loading of extracellular vesicles using microbubble-assisted ultrasound

Yuana Yuana[a], Kim van der Wurff-Jacobs[a], Banuja Balachandran[a], Linglei Jiang[b] and Raymond Schiffelers[c]

[a]Division Imaging, UMC Utrecht, The Netherlands, Utrecht, Netherlands; [b]Department of Clinical Chemistry and Haematology, UMC Utrecht, The Netherlands; [c]Laboratory of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, Netherlands

Introduction: Development of extracellular vesicles (EVs) as nanocarriers for drug delivery relies on loading a substantial amount of drug into EVs. Loading has been done from the simplest way by co-incubating the drug with EVs or producer cells until using physical/chemical methods (e.g. electroporation, extrusion, and EV surface functionalization). We use physical method combining gas-filled microbubbles with ultrasound known as sonoporation (USMB) to pre-load drug in the producer cells, which are eventually loaded into EVs.

Methods: Cells were grown overnight in 0.01% poly-L-lysine coated cell culture cassette. Prior to USMB, cells were starved for 4 h. Treatment medium containing microbubbles and 250 µg BSA-Alexa Fluor 488 as a model drug was added to the cells grown in the cassette. Cells were exposed directly to pulsed ultrasound (10% duty cycle, 1 kHz pulse repetition frequency, and 100 µs pulse duration) with up to 845 kPa acoustic pressure. After USMB, cells were incubated for 30 min and then treatment medium was removed.

Cells were washed and incubated in the culture medium for 2 h. Afterward, EVs in the conditioned medium were collected and measured.
Results: Cells took up BSA-Alexa Fluor 488 after USMB treatment as measured by flow cytometry. These cells released EVs in the conditioned medium which were captured by anti-CD9 magnetic beads. About 5% of the CD9-positive EVs contained BSA-Alexa Fluor 488. The presence of CD9-positive EVs containing BSA also were confirmed by immunogold electron microscopy.
Summary/Conclusion: USMB serves as a tool to preload the model drug, BSA-Alexa Fluor 488, endogenously and to produce EVs loaded with this model drug. USMB setup, incubation time, and type of drugs will be investigated to further optimize the production of drug-loaded EVs and to explore possible application for in situ drug delivery system.
Funding: This research is funded by Focused Ultrasound Foundation.

OS23.05

Extracellular Vesicles for new Molecular Insight to Biomolecular Interactions

Tamas Beke-Somfai[a], Priyanka Singh[v], Imola Szigyarto and Zoltan Varga[c]

[a]PI, Budapest, Hungary; [b]Ms, Budapest, Hungary; [c]Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary

Introduction: The potential of extracellular vesicles (EVs) to revolutionize the diagnosis and therapy of various diseases has been realized and thus it is an extensively studied direction. However, EVs are also in the size range suitable for membrane biophysics, while they preserve the complex composition of a biological bilayer. Consequently, they are optimal for monitoring the structure, orientation and function of biomolecules associated to EVs.
Methods: The investigated red blood cell-derived vesicles (REVs) were isolated from blood using a standard protocol and purified using size-exclusion chromatography. REVs were subjected to IR, CD and flow-Linear Dichroism spectroscopy, freeze-fracture Transmission Electron Microscopy as well as Dynamic Light Scattering.
Results: Here we demonstrate that polarized light spectroscopy techniques can provide important information on REVs and molecules inserting into their bilayer. Flow-linear dichroism (flow-LD) measurements show that EVs can be oriented by shear force, insight into properties of oriented macromolecules in the vesicles. The Soret-band of the LD spectra demonstrates that hemoglobin molecules are oriented and associated to the lipid bilayer in freshly released REVs [1].

Further on, we selected three different antimicrobial peptides (AMPs), CM15, melittin and gramicidin and investigated their interactions with REVs using a diverse set of techniques. The peptide-membrane interactions reveal several novel function of AMPs, including their ability to remove associated proteins from the surface of REVs (Figure 1).

[1] I. Cs. Szigyártó, R. Deák, J. Mihály, S. Rocha, F. Zsila, Z. Varga, T. Beke-Somfai. Flow-alignment of extracellular vesicles: structure and orientation of membrane associated biomacromolecules studied with polarized light. ChemBioChem. 2018;19:545–551
Summary/Conclusion: In conclusion, EVs provide excellent opportunities to better understand the function and mechanism of natural membrane active biomolecues.
Funding: This work was funded by the Momentum programme (LP2016-2), by the National Competitiveness and Excellence Program (NVKP_16-1-2016-0007) and BIONANO_GINOP-2.3.2-15-2016-00017. The János Bolyai Research Scholarship (Z.V.) is greatly acknowledged.

Symposium Session 24: Mechanisms of EV Delivery
Chairs: Pieter Vader; Hang Hubert Yin
Location: Level B1, Hall B

13:00–14:30

OS24.01

State of the art microscopy for live cell study of the extracellular vesicle-mediated drug delivery

Ekaterina Lisitsyna[a], Kaisa Rautaniemi[a], Heikki Saari[b], Timo Laaksonen[a], Marjo Yliperttula[b] and Elina Vuorimaa-Laukkanen[a]

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Introduction: Extracellular vesicles (EVs) provide a compelling alternative for targeted drug delivery due to the unique set of their properties: (1) natural protection of EV content from degradation in the circulation; (2) EVs' intrinsic cell targeting properties and (3) innate biocompatibility. However, their mechanisms of interacting with living cells are poorly understood.

Methods: Microvesicles (MVs) and exosomes (EXOs) derived from prostate cancer cells were studied. The EVs were passively loaded with the conjugate of cancer drug Paclitaxel (Ptx) and fluorescent probe Oregon Green (OG). Ptx-OG EVs were applied to the cells autologously and imaged by fluorescence lifetime microscopy (FLIM). Simultaneous labelling of cell organelles with the FRET pairs to OG was done to utilize FLIM in combination with Foerster resonance energy transfer (FLIM-FRET). Time-resolved fluorescence anisotropy imaging (TR-FAIM) was applied for the first time to study the EV-based drug delivery. Confocal microscopy was used as a standard method of live cell imaging.

Results: By FLIM, we show distinct cellular uptake mechanisms for EXOs and MVs loaded with the drug-dye conjugate Ptx-OG. We demonstrate differences in intracellular behaviour and drug release profiles of Ptx-containing EVs in correlation with the intracellular position. Based on FLIM and confocal data we suggest that EXOs deliver the drug mostly by endocytosis while MVs enter the cells by both endocytosis and fusion with the cell membrane. TR-FAIM shows that Ptx-OG binds some intracellular target inside the cell that is in accordance with the known fact that Ptx interacts with microtubules network.

Summary/Conclusion: This research offers new real-time methods to investigate EV kinetics with living cells and complements the existing techniques. The findings of the study improve the current knowledge in exploiting EVs as drug delivery systems.

Funding: The research is funded by Academy of Finland projects 311362 and 258114.

OS24.02

Fusion of extracellular vesicles (EVs) and delivery of internal EV cargos to host cells is dependent upon circulating or endogenous viral envelope proteins

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Introduction: Extracellular vesicles (EVs) contain proteins and small RNAs that are posited to mediate cell-to-cell communication; however, the precise molecular mechanisms of EV fusion to host cells and delivery of internal cargos remains poorly defined. Delivery of internal EV cargos to target cells requires fusion between the EV and cell membranes; otherwise, the EV and its contents are degraded by lysosomal enzymes. In this study, we probed the molecular mechanisms of EV fusion by adapting and employing a validated and powerful viral fusion assay.

Methods: EVs were produced in HEK 293T cells and labelled with beta-lactamase (BlaM) by overexpression or with BlaM-CD9/CD63/CD81 chimeric proteins. In some conditions, the HEK 293T cells were also transfected with plasmids encoding viral envelope glycoprotein (Env) proteins. EVs were isolated by ultracentrifugation and size exclusion chromatography, characterized by TEM imaging, and titered with microBCA assay. To test EV fusion, EVs were added to target cells containing CCF2-AM FRET dye. Fusion was measured by flow-cytometric evaluation of CCF2-AM dye cleavage by BlaM.

Results: EVs produced in the absence of viral Env showed no evidence of fusion with target cells. In contrast, EVs produced in cells co-transfected with vesicular stomatitis virus Env (VSV-G) were highly fusogenic even at low doses. EV fusion was dependent on the presence of functional viral Env machinery,

either from actively circulating viruses – including VSV-G, rabies, influenza, and mokola viruses – or from human endogenous retroviruses (HERVs) Env proteins – such as syncytin-1.

Summary/Conclusion: EVs produced in the absence of viral Env machinery are poorly fusogenic and are unlikely to be efficient mediators of cell-to-cell communication via the delivery of EV contents to the cytoplasm. In contrast, viral Env proteins significantly enhance EV fusogenicity, suggesting that EV fusion and communication may occur and play a significant role during viral infections. Furthermore, cells expressing the HERV Env syncytin-1 – including many human cancers – also give rise to fusogenic EVs that may contribute to tumour establishment, growth, and metastasis. These findings suggest that blocking syncytin-mediated EV fusion may be an effective strategy to block EV communication in human cancers.

OS24.03

Preferential accumulation of copper-free click chemistry-modified exosomes to own pancreatic xenograft *in vivo*

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Introduction: Pancreatic cancer (PC) is one of the deadliest malignancy with few effective approaches available for early diagnosis or therapy. Exosomes (Exo) as one type of extracellular vesicles are currently being investigated as potential theragnostic tools in cancer. However, it is not yet well-understood how Exo are taken up by PC cells. This work aims to study the Exo dosimetry and preferential Exo-cell affinity in PC cells *in vitro* and *in vivo* for exploitation of Exo-based delivery of therapeutics.

Methods: Exo are isolated by sucrose cushion ultracentrifugation and characterized for exosomal marker expression, number, purity and shape. Exo were fluorescently labelled by copper-free click chemistry to enable uptake quantification in cells using the Design of Experiments (DoE) approach. Cellular uptake of Exo was investigated using flow cytometry and confocal microscopy. Factors studied are donor Exo source, dose, receipt cell type, and incubation time. Responses identified are Exo "Taken up numbers" and "Percentage uptake" per cell. Candidate PC Exo uptake was then assessed *in vivo* and compared between PC and melanoma xenograft models in NSG mice following intravenous administration.

Results: Cellular uptake of Exo was time- and dose-dependent profiles. PC derived PANC-1 Exo showed significantly higher and not saturable uptake in PANC-1 cells compared to B16-F10 Exo (cancer-derived) and HEK-293 Exo (non-cancer derived) which showed lower and saturable uptake profile at 24 h. *In vivo* biodistribution studies of PANC-1 Exo in subcutaneous PC xenograft further confirmed that PANC-1 Exo favoured accumulation in PC tumours over melanoma (B16-F10) tumours.

Summary/Conclusion: A simple and highly efficient surface modification approach via click chemistry was developed enabling both *in vitro* and *in vivo* tracking of Exo. DoE modelling predicted PC cells' preference to PC-derived Exo which was confirmed also *in vivo*. This Exo dosimetry study could facilitate a rationalized approach in Exo-based therapeutics for treatment of cancer in pre-clinical studies.

Funding: The K. C. Wong Education Foundation and The Marie Skłodowska-Curie actions, European Commission "Horizon 2020", EU (H2020-MSCA-IF-2016)

OS24.04

Specific transfer of hollow gold nanoparticles within exosomes is determined by the exosome origin

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Introduction: Exosomes are considered key elements for communication between cells but very little is known about the mechanisms and selectivity of the transference processes involving exosomes released from different cells.

Methods: In this study we have investigated the transfer of hollow gold nanoparticles (HGNs) between different cells when these HGNs were loaded within exosomes secreted by human placental mesenchymal stem cells (MSCs). These HGNs were successfully incorporated in the MSCs exosome biogenesis pathway and released as HGNs-loaded exosomes, by using time-lapse microscopy and atomic emission spectroscopy

Results: Those studies allowed us to demonstrate the selective transfer of the secreted exosomes only to the cell type of origin when studying different cell types

including cancer, metastatic, stem or immunological cells.

Summary/Conclusion: In this study we demonstrate the selectivity of *in vitro* exosomal transfer between certain cell types and how this phenomenon can be exploited to develop new specific vectors for advanced therapies. We show how this preferential uptake can be leveraged to selectively induce cell death by light-induced hyperthermia only in cells of the same type as those producing the corresponding loaded exosomes. We describe how the exosomes are preferentially transferred to some cell types but not to others, thus providing a better understanding to design selective therapies for different diseases.

Funding: We thank the ERC Consolidator Grant program (ERC-2013- CoG-614715, NANOHEDONISM) for the financial support, and CIBER-BBN, financed by the Instituto de Salud Carlos III.

OS24.05

A high-throughput screen for functional extracellular vesicles

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Introduction: Prions are infectious protein aggregates that self-propagate and infect naïve cells by direct cell contact or via secreted vesicles. Several lines of evidence argue that also protein aggregates associated with common neurodegenerative diseases can intercellularly propagate their aggregated states in a prion-like manner. Thus, targeting extracellular vesicles (EVs) has potential clinical implications for neurodegenerative diseases. We have developed a mouse neuroblastoma cell-based assay to identify compounds that modulate exosome uptake and subsequent protein aggregate formation in recipient cells. In this novel cell-based assay, we take advantage of the non-toxic Saccharomyces cerevisiae prion domain Sup35NM that forms self-templating protein aggregates in mammalian cells capable of spreading through cell cultures. The addition of fibrils produced from bacterially expressed Sup35NM to cells expressing soluble NM efficiently induces appearance of NM aggregates which are faithfully inherited by daughter cells. Importantly, EVs released from donor cells containing NM aggregates are infectious and induce the aggregation of soluble NM-GFP in recipient cells after 12 h incubation time. We here introduce a high throughput assay to screen for functional EVs that trigger NM reporter protein aggregation in target cells.

Methods: We have developed a quantitative high-throughput screen assay to identify modulators (inhibitors and activators) on exosome uptake. The read-out of this functional EV assay is the percentage of recipient cells with induced NM-GFP aggregates.

Results: A total of 4135 small molecules were screened from three well-defined compound libraries (LOPAC, TOCRIS and SELLECKCHEM). Thirty-three inhibitors and 35 activators were found to decrease or increase the EV-mediated aggregate induction in recipient cells, respectively. Lead compounds identified in this screen affect active and selective EV uptake in recipient cells.

Summary/Conclusion: We successively developed a cell-based assay for functional extracellular vesicles and performed high-throughput screening to identify the mechanisms of active extracellular vesicle uptake. I will present some interesting findings out of the screen.

Symposium Session 25: EVs in Neurological Diseases
Chairs: Andrew Hill; Yiyao Huang
Location: Level B1, Hall A

13:00–14:30

OS25.01

Circulating extracellular vesicles of astrocytic origin carry neurotoxic complement in Alzheimer's disease

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Introduction: Recent research has documented the role of reactive astrocytes in neuroinflammation in Alzheimer's disease (AD), and of Extracellular vesicles (EVs) in the transneuronal propagation and seeding of Aβ, tau and other pathogenic protein mediators. However, the mechanisms underlying the initial induction and propagation of neurodegeneration in AD remain elusive. In our Lab, we have pioneered the isolation of neuronal- and astrocytic-derived EVs (NDEVs, ADEVs) from peripheral blood and have found that, in AD patients, NDEVs contain pathogenic Aβ and tau, whereas ADEVs contain high levels of potentially toxic complement. Based on these observations we hypothesized that ADEVs and/or NDEVs circulating in the plasma of AD patients are neurotoxic.

Methods: We isolated plasma ADEVs, NDEVs and CD81+ EVs from patients with sporadic AD and age-matched controls. To assess their ability to induce neurotoxicity, we used them to incubate cultures of rat cortical neurons and human iPSC-derived neurons. We studied neuronal viability using the MTT assay and neurite density quantification; necrosis using fluorescent detection of EthD-1; and apoptosis using caspase 3/7 assays *in vitro*. We used the physiologic inhibitor of the terminal complement pathway CD59 in rescue experiments. In evolving vivo experiments, we perform hippocampal injections in rats and study neurodegeneration and induction of Aβ and tau pathology.

Results: Neurons incubated with NDEVs and ADEVs from AD patients exhibited significantly decreased neurite density, cell viability, and increased necrotic and apoptotic cell death, compared to neurons treated with control EV subpopulations (CD81+, total EVs) from patients or ADEVs or NDEVs from control participants. Blocking the formation of the complement Membrane Attack complex with CD59 rescues the toxicity.

Summary/Conclusion: This is the first demonstration that blood-borne EVs from AD patients are neurotoxic through a complement-mediated mechanism. These findings indicate a novel mechanism for induction and perhaps propagation of neurodegeneration in AD through circulating EVs with important therapeutic implications.

Funding: This research was supported entirely by the Intramural Research Program of the National Institute on Aging, NIH.

OS25.02

Platelet extracellular vesicles as first liquid biopsy biomarkers to diagnose acute ischaemic stroke

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Introduction: Acute ischemic stroke is the second most common cause of death in Europe, accounting for almost 1.1 million deaths annually. Diagnosis of stroke relies on neurologic deficits and brain imaging. Because time is brain, stroke is preferably already diagnosed in the ambulance, which requires a liquid biopsy biomarker. Our aim is to determine whether EVs from platelets, leukocytes and endothelial cells can be used as biomarker to diagnose stroke.

Methods: The study was approved by the medical ethics committee. Venous blood was collected at days 1 (acute phase) and 7 (late phase) after the onset of stroke from fasting patients (n = 19, mean age 53.8 ± 5.4 years, 55% male) and controls (patients with Parkinson or Alzheimer disease, n = 9, mean age 57.1 ± 3.2 years, 53% male). Flow cytometry (Apogee A60 Micro) was used to determine plasma

concentrations of EVs labelled with antibodies for activated platelets (CD61, CD62p; PEVs), leukocytes (CD45; LEVs) and endothelial cells (CD146; EEVs). Flow cytometry data files were processed using in-house developed, automated software (MATLAB R2018a), enabling flow rate stabilization, diameter and refractive index determination, MESF calibration, fluorescent gate determination and application, and statistics reporting. To standardize and differentiate EVs from small platelets and lipoproteins, only events between 200 and 700 nm and with a refractive index <1.42 were included.

Results: Concentrations of PEV were elevated in stroke patients compared to controls, both at day 1 and day 7 (p = 0.035, p = 0.059, respectively). Concentrations of LEVs were comparable at day 1 (p = 0.83) and decreased at day 7 (p = 0.059), whereas concentrations of EEVs decreased at day 1 (p = 0.048) and normalized to control levels at day 7 (p = 0.91).

Summary/Conclusion: Concentrations of platelet EVs are elevated in patients with stroke both at day 1 and day 7, compared to controls. In follow-up studies, we are going to validate platelet EVs as the first liquid biopsy biomarker to diagnose ischemic stroke. Concentrations of LEVs and EEVs fluctuate between day 1 and day 7 after stroke, likely reflecting activation of immune system and endothelium following brain damage.

Funding: Polish National Science Centre (2017/25/N/NZ5/00545)

OS25.03

Circulating extracellular vesicles as a novel source of biomarkers for diagnosis and monitoring of neurological diseases

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Introduction: One of the main hurdle in clinical approach to neurological diseases is the lack of easily accessible and sensitive biomarkers. Brain-derived extracellular vesicles (EVs) circulate in blood, cross the blood–brain barrier and are involved in the onset and progression of neurological disorders, but reliable, sensitive and reproducible methods that can cope with their nanoscale dimensions are still lacking.

We propose to validate brain-derived EVs as new biomarkers of Stroke, Alzheimer's disease (AD) and Parkinson's disease (PD) by using biophotonics-based techniques as Surface Plasmon Resonance imaging (SPRi) and Raman Spectroscopy (RS).

Methods: EVs were isolated by size exclusion chromatography and ultracentrifugation from plasma and serum of Stroke, AD and PD patients and healthy controls recruited according to a protocol approved by the medical Ethics Committee. We developed an SPRi antibody array to separate and characterize plasma EVs of different neural origins. In parallel, RS was applied to serum EVs in order to obtain a snapshot of their biochemical profiling. Statistical analysis was applied for the comparison of SPRi and Raman data from healthy subjects and neurological patients.

Results: After the successful SPRi detection of EVs from neurons, oligodendrocytes, astrocytes and microglia, the quantification of specific surface molecules related to pathological or recovery processes has revealed variations of EVs specific content during a disease. Moreover, the bulk characterization of EVs by RS demonstrated the presence of EVs loaded with atypical cargoes when compared to healthy controls.

Summary/Conclusion: Our results provide support for using EVs as biomarkers for monitoring the progression of Stroke, AD and PD, suggesting the possibility to use the SPRi-biosensor and Raman fingerprint to identify and verify the neuro-pathological or recovery processes ongoing in neurological patients.

Funding: This study was supported by the Italian Ministry of Health.

OS25.04

Extracellular vesicles of Alzheimer's disease patients as a biomarker for disease progression

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Introduction: Introduction: Alzheimer's disease (AD) is progressive irreversible neurodegenerative pathology and the most common cause of degenerative dementia. AD becomes symptomatic only after brain changes occur over years.Accumulating evidence suggests that extracellular vesicles (EVs) that contain cytokines and microRNA are involved in the regulation of inflammation. The current study aimed

to characterize the EVs of AD patients as a biomarker for disease progression.
Methods: Blood samples were collected after obtaining signed informed consent (No. 0462-14-RMB) from 39 AD patients at three stages of disease severity and from 14 healthy controls (HC). Cerebrospinal fluid was collected from five patients and three HC. EV size and concentration were studied by Nano-tracking analysis. Membrane antigens were characterized by their cell origin as defined by flow cytometry. EV protein contents were screened by protein array, and miRNA content was screened by Nano-string technology and validated by RT-PCR.
Results: The AD patients' EVs were significantly smaller and the levels of neural cell markers were higher than EVs obtained from HC. Moderate or severe AD patients' EVs had a significantly higher level of the Myelin oligodendrocyte glycoprotein (MOG), compared to the EVs obtained from patients with mild AD ($P = 0.0002$ and $P = 0.036$). Levels of the EVs that expressed the axonal glycoprotein CD171 were significantly higher in the patients with severe AD compared to HC ($P = 0.0066$), possibly indicating injured apoptotic neural cells. There was also a significant increase in EVs originating from endothelial cells (labelled with CD31+ CD41-, $P = 0.0115$ and with CD144, $P = 0.0276$) in patients with moderate AD compared EVs obtained from the HC. A >2-fold increase was measured in the content of inflammatory cytokines (TNFα, IL8, IL-2, IFNγ) as was a >50% reduction in growth factors (FGF, EGF VEGF) and their receptors in the EVs of moderate AD patients. miR-146a-5p and several other miRNAs obtained from the EVs of severe AD patients had significantly low levels compared to HC.
Summary/Conclusion: The neural and endothelial damage severity as reflected by AD patients' EVs (antigen profiles cytokine and miRNA) may serve as a biomarker for disease dynamics.

OS25.05

Novel Blood-derived Extracellular Vesicle-based Biomarkers in Alzheimer's Disease by the Proximity Extension Assay

Jonas E. Nielsen[a], Kamilla Pedersen[b], Karsten Vestergård[c], Søren Kristensen[a] and Shona Pedersen[a]

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Introduction: Biomarkers capable of identifying complex pathways contributing to neuropathological development, especially in the early stages of Alzheimer's disease (AD), are lacking. Such biomarkers could be present in easily available fluids, such as blood, due to the breakdown of the blood–brain barrier (BBB) early in AD. However, the identification of specific and sensitive blood-based biomarkers is a challenging task. Therefore, extracellular vesicles (EVs) may provide a window into AD etiology and therapeutic targets, as brain-derived EVs have been shown to cross the BBB and are present in blood. As biomarkers, proteins are a potential source of relevant information relating to biological function. Thus, we investigated a subset of proteins hypothesized to be involved in neurological processes in plasma and EV samples using the Proximity Extension Assay (PEA).
Methods: EVs were isolated from platelet poor plasma from 10 healthy controls (HC), 10 patients with Mild Cognitive Impairment (MCI) and 10 patients with mild/moderate AD. Isolation was performed using centrifugation at 20.000 xg, 1 h, 4°C with a subsequent washing of the pellet at the same g-force. For the characterization of the EV isolates, Nanosight and western blotting (CD9) are performed. A neurology panel of 92 biomarkers were assessed in plasma and EVs using the PEA. Written informed consent was obtained from all study participants and the study was approved by The North Denmark Region Committee on Health Research Ethics (N-20150010).
Results: PEA showed no significant difference of protein levels comparing the three groups for the plasma samples. Interestingly, EV samples showed four statistically significant proteins; Siglec-9, CLM-1, CLM-6 and CD38, which were less expressed in the MCI and AD groups compared with the HC group with a false discovery rate adjusted *p*-values of 0.014, 0.024, 0.035 and 0.031, respectively. These proteins have been documented to be involved in neurotoxicity protection and inflammatory regulation.
Summary/Conclusion: Our preliminary results demonstrate that EVs, compared to plasma, hold potential as candidate diagnostic biomarkers in AD.

OS25.06

Proteomic and transcriptomic profiting of extracellular vesicles isolated from immune-stimulated human primary astrocytes

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Introduction: Astrocytes are abundant glial cells in the central nervous system that provide supportive neuronal functions. They have critical roles in regulating

neuronal activities in response to pro-inflammatory factors in neurodegenerative diseases. Exosomes, typically 50–150 nm in size extracellular microvesicles, are known to carry a large diversity of molecules such as proteins and RNA species that can modify the physiology of recipient cells. Here, we hypothesized that astrocyte-derived exosomal proteins are regulated when exposure to pro-inflammatory factors, thus transported to control neuronal function and plasticity.

Methods: We performed a quantitative proteomic and transcriptomic analysis of exosomes purified from human primary astrocytes with or without interleukin 1-β (IL1-β) stimulation *in vitro*. Exosome-enriched fractions were purified by size-exclusion columns. The total proteins isolated from the EVs were run on 1D SDS-PAGE and mass spectrometry. miRNA was isolated from EVs and subjected to Affymetrix miRNA 4.0 Array. The data are subjected to bioinformatic analysis and validation for select molecules.

Results: A total of 539 common proteins were identified. IL1-β-stimulated astrocytes enhanced the cargo load of proteins in the EVs. IL-1b stimulation induced activation of immune response and modulation of cell adhesions. The EVs from resting astrocytes play a role in protein metabolism, cell growth and maintenance. Finally, similar proteomic results were also obtained from exosomes derived from astrocytes cultured in serum-free media with IL1-β stimulation, further validating the alteration of exosomal proteins in activated astrocytes which can be transferred to control neuronal function and plasticity.

Summary/Conclusion: Our finding will be helpful to elucidate the pathophysiological functions of astrocyte-derived exosomes in regulating neuronal networks and provide new insights into the diagnostics and therapeutics of inflammatory diseases.

Funding: NIH 1R01AG054672, 1R56AG057469 and 1RF1AG054199 (TI), 5R24HD0008836

Saturday Poster Session
PS01: Engineering and Loading EVs
Chairs: Hang Hubert Yin; Antonella Bongiovanni
Location: Level 3, Hall A

15:00–16:00

PS01.01

Targeting prostate cancer via PSMA-peptide decorated exosome-mimetics

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Queen's University Belfast, Belfast, UK

Introduction: Prostate cancer (PC) is the most common type of cancer and the second cause of death in men worldwide. A range of effective anticancer drugs have been used to treat advanced PC, however, their systemic toxicity has limited their clinical use. Therefore, there is an unmet need to develop novel strategies to deliver cancer therapeutics to PC tissues. Exosomes are nanosized, cell-derived vesicles that carry proteins and RNAs for intercellular communication. They could also deliver their cargo across the plasma membrane and delay premature drug transformation and elimination. Exosomes have shown an intrinsic homing ability to a wide range of cells. Furthermore, a new approach has been proposed to combine the intrinsic homing ability of exosomes with active targeting to enhance their tumour accumulation. In the present work, we report the development of novel prostate-specific membrane antigen (PSMA)-targeted exosome-mimetics (EMs) for advanced PC.
Methods: Stably transfected PSMA-peptide expressing monocytes U937 cell line was established. PSMA-targeted EMs were prepared by serial extrusion of the transfected U937 monocytes. The PSMA-targeted EMs were characterized by dynamic light scattering, nanoparticle tracking analysis, transmission electron microscopy, bicinchoninic acid assay and western blotting. Furthermore, the binding of the PSMA-targeted EMs to the recombinant human PSMA protein was confirmed by ELISA. Their drug loading capability was assessed by loading doxorubicin and its derivatives. Next, *in vivo* biodistribution and safety studies of targeted EMs were carried out in C4-2B and PC3-tumour-bearing mice.
Results: The engineered EMs exhibited high protein yield, good drug loading and exosome markers expression. The expression of PSMA targeting peptide and its binding to PSMA receptors was confirmed *in vitro*. Finally, successful tumour accumulation of PSMA-targeted EMs was achieved *in vivo* with the absence of *in vivo* toxicity.
Summary/Conclusion: Our engineered PSMA-targeted EMs, could offer a promising drug delivery system for PC, based on its drug loading capacity, tumour targeting and safety *in vivo*.
Funding: Rosetrees Trust studentship (A1108), PCUK (CDF-12-002 Fellowship) and EPSRC (EP/M008657/1).

PS01.02

Improved loading of plasma-derived extracellular vesicles to encapsulate antitumour miRNAs

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Introduction: Extracellular vesicles (EVs) are particles released by cells that carry a complex cargo of molecules and mediate intercellular communication. Recently, they have raised great interest as drug delivery systems and several engineering methods are currently under investigation. Numerous factors, however, influence the transfection yield, including protocol variability and EV damage.
Methods: The electroporation was investigated as method to directly load miRNAs in plasma-derived EVs. Different parameters (voltage and number of pulses) were compared for their effect on EV morphology and loading capacity of a synthetic miRNA, cel-39, including miRNA enrichment in EVs and its transfer to target cells. Next, analyses were performed to evaluated the transfection effect on EV endogenous cargo and the exogenous miRNA protection from RNAse degradation. Then, EVs were loaded with antitumour miRNAs and their pro-apoptotic effect was evaluated on a cell line of hepatocellular carcinoma, HepG2 cells.

Results: The comparison of different electroporation settings demonstrated the importance of choosing the more appropriate protocol parameters to obtain an efficient EV transfection yield, understood as both molecules loading and EV damage. In particular, we observed the superiority of one electroporation protocol (using 750 Volt and 10 pulses) that allowed the most efficient miRNA packaging and transfer to target cells, without structurally damaging EVs. The most efficient electroporation protocol was also proven to allow a more efficient miRNA loading in respect to incubation, better protecting miRNA from enzymatic digestion. In addition, our findings suggested that electroporation preserved the naïve EV cargo, including RNAs and proteins, and did not alter their uptake in cells. EVs engineered with antitumor miRNAs (miR-31 and miR-451a) successfully promoted the apoptosis of HepG2 cells, downregulating their target genes related to apoptotic pathways.

Summary/Conclusion: In conclusion, our findings indicate an efficient and functional miRNA encapsulation in plasma-derived EVs following an electroporation protocol that preserves EV integrity.

Funding: Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), Unicyte AG (Switzerland)

PS01.04

Development of a platform for exosome engineering using a novel and selective scaffold protein for surface display

Kevin Dooley, Ke Xu, Sonya Haupt, Shelly Martin, Russell McConnell, Nuruddeen Lewis, Christine McCoy, Chang Ling Sia, Jorge Sanchez-Salazar, Nikki Ross, Rane Harrison, Bryan Choi, Damian Houde, John Kulman and Sriram Sathyanarayanan

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Introduction: Membrane proteins preferentially partitioned into exosomes can be co-opted to display pharmacologically active molecules on the exosome surface, which is an important strategy for maximizing the potential of therapeutic exosomes. Previously published approaches have relied on "canonical" scaffolds including multi-pass transmembrane tetraspanins (CD9/CD63/CD81), LAMP2B, or non-exosomal domains such as pDisplay or GPI anchors. We sought to identify novel scaffolds that enable more uniform, higher density surface display of structurally and biologically diverse molecules.

Methods: Proteomic analysis of stringently purified exosomes led to the identification of highly abundant and unique exosomal proteins, including a single-pass transmembrane glycoprotein (Protein X, PrX) belonging to the immunoglobulin superfamily. Protein X and fragments thereof were expressed in a cell line and the minimum PrX domain requirements for exosomal enrichment were determined. Leveraging PrX as a scaffold for exosome surface display, we developed our engEx platform to generate engineered exosomes functionalized with a variety of pharmacologic payloads including enzymes, antibodies, type I cytokines and TNF superfamily members. Biological activity of these engineered exosomes was assessed in an array of *in vitro* assays and compared to previously described scaffolds.

Results: Stable expression of PrX in an exosome producing cell line resulted in 200-fold enrichment of PrX on secreted exosomes. Interestingly, overexpression of PrX structural paralogs did not result in similar levels of enrichment, suggesting PrX is unique. Exosomes expressing PrX-GFP exhibited 100-fold increase in relative fluorescence compared to LAMP2B and pDisplay GFP fusions. Similar levels of high-density expression were achieved with a variety of topologically diverse therapeutic proteins fused to full-length or truncated forms of PrX. Exosomes engineered to display IL7, CD40 ligand, IL12 and antibody fragments via PrX fusion exhibited up to 1500-fold improvement in potency compared to previously described scaffolds.

Summary/Conclusion: This work demonstrates the potential of our engEx platform to generate novel exosome therapeutics, specifically through high density surface display mediated by PrX.

PS01.05

Leptin-loaded macrophage-derived exosome: high-efficiency loading method and its properties

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Introduction: Exosome, one of extracellular vesicles, is considered to be an important player in intercellular communication. Application of exosome to drug delivery system is expected to target specific cells. Especially macrophage-derived exosome is known to cross blood–brain barrier (BBB) and deliver its cargo after intravenous administration. Leptin is hormone to regulate energy balance by inhibiting hunger, and leptin receptor is located on neurons of hypothalamus. Drug delivery system of leptin to brain is anticipated because leptin transporter at BBB is known to be impaired in obesity models. However, it has been challenging to load

enough amount of protein drugs into exosome without changing its original properties.

Purposes of this research are to develop leptin-loading method into exosome with high efficiency and to evaluate its physicochemical and biological characteristics.

Methods: Exosome was isolated from IC-21 (mouse macrophage) cells by an ultracentrifuge method. Particle-size distribution of the exosome was measured by Nanoparticle Tracking Analysis. Expression of exosome-marker protein was confirmed by Simple Western. Leptin was loaded into the exosome by using a probe sonicator, and free leptin was removed by gel filtration chromatography. Loaded amount of leptin was measured by ELISA. Release profile of leptin from the exosome was evaluated in mouse serum at 37° C. In order to evaluate protection ability of exosome formulation against protease, the leptin-loaded exosome was treated with pronase and remained leptin was quantified. Stability of the exosome was also investigated.

Results: IC-21 derived exosome had 100–110 nm of mean size and contained exosomal markers, such as Alix and Rab11A. Size distribution and exosomal marker level of the leptin-loaded exosome prepared under optimized condition were similar to those of bare exosome. Drug-loading efficiency was 7% in this condition. Although ~50% of leptin burst from the exosome after release study and ~70% of leptin was degraded by protease challenge test, the other leptin was considered to be retained in the exosome. Particle-size distribution and leptin concentration of the exosome were stable at 4°C for 1 month.

Summary/Conclusion: This methodology to load protein drugs into exosome is promising strategy for its drug delivery application.

PS01.06

Characterization and *in vivo* imaging of mesenchymal stem cells derived extracellular vesicle

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Introduction: Mesenchymal stem cells (MSCs) are multipotent stromal cells which show the great potential in tissue engineering, regenerative medicine and the treatment of various diseases. Deep into mechanisms, paracrine effect has been reported to be the major role in MSC therapy. Further, extracellular vesicles (EVs) are reportedly the major player mediating the therapeutic and paracrine effects of MSCs. With the rapid increase of attention and being of great potential as a future medical regimen for human disease, the information of fate and behaviour of EVs in the living subject should be urgently gathered. However, investigators still have not developed an effective method to monitor the *in vivo* behaviour of EVs. Therefore, here in our study, EVs derived from Wharton’s jelly MSCs were isolated, characterized and radiolabeled with 111In-oxine followed by biodistribution study and *in vivo* SPECT/CT imaging.

Methods: Conditioned medium was collected followed by exosome isolation using Exo-Prep kit (Hansa BioMed) followed by purification with PD-10 columns and 100 kDa concentration. Expression of EVs specific proteins CD63 and HSP70 was verified by western blot. Morphology and size were characterized by transmission electron microscopy nanoparticle tracking analysis (NTA). For radiolabeling, EVs were incubated with 111In-oxine in PBS at 37oc for 1 h followed by purification and further characterization. Biodistribution and *in vivo* SPECT/CT imaging of 111In-oxine- labelled EVs were performed at 1, 3, 6 and 24 h after intravenous injection into C57BL/6 mice.

Results: CD63 and HSP70 expression were observed on EVs as well as 111In-oxine-EVs. Radiochemical purity of 111In-oxine-EVs as higher than 90% and remained stable for at least 48 h. Result of biodistribution showed that 111In-oxine-labelled EVs accumulated in liver, spleen, bone marrow and cleared rapidly from the circulation. *In vivo* SPECT/CT imaging of 111In-oxine-labelled EVs showed high accumulation in liver, bone, spleen and liver, but not in brain and circulation.

Summary/Conclusion: In this study, we have preliminarily demonstrated the feasibility of *in vivo* tracking of MSC- derived EVs labelled with 111In-oxine. Further investigation is still needed and underway to monitor the *in vivo* fate and behaviour of EVs.

PS01.07

EVs as siRNA delivery vehicles for functional knockdown in cells

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Introduction: Extracellular vesicles (EVs) mediate cell-to-cell communication by delivering cargo, composed of nucleic acids, proteins and various other molecules, from secreting cells to specific tissues and recipient

cells. This method of cellular delivery has generated great interest in targeted delivery of therapeutics, such as chemical probes, proteins and RNA. In particular, EV RNA has gained increasing attention not only in biomarker development but also in the regulation of gene expression in cells. The delivery of siRNA by EVs is one method to induce gene silencing. Here we developed and optimized a method of loading EVs with siRNA using a chemical transfection reagent.

Methods: Isolated EVs from tissue culture media were loaded with siRNA using a chemical transfection reagent. Various compositions of the chemical, as well as molar ratio between the chemical and siRNA, temperatures and EV concentrations were examined to determine optimal conditions. The transfected EVs were added onto cells and incubated for 24–48 hrs. After the incubation period, the cells were imaged for fluorescence and cell morphology changes. Then, the cells were harvested, lysed and analysed by Western blot (protein level). Additionally, cell lysates RNA was isolated and analysed by RT-qPCR.

Results: Fluorescent imaging showed a dose-dependent incorporation of control Cy3-labelled siRNA into cells. The cell nuclear morphology was examined after delivery of ECT2 and TOR1AIP1 siRNA. In addition, RT-qPCR and Western blotting analyses were used to measure the level of knockdown of the housekeeping gene, HPRT. CCK8 assay was used to determine cell viability after delivery of EVs loaded with control siRNA and other siRNAs.

Summary/Conclusion: Our results provide a specific and efficient approach for loading siRNA directly into EVs for the implementation of targeted gene silencing.

PS01.08

Development of an *in vivo* extracellular vesicle-based peptide library screening tool

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Introduction: Extracellular vesicles (EVs) including exosomes and microvesicles are heterogenous population of membrane-bound vesicles with cargos including protein, lipids and nucleic acids of DNA and RNA species. Recently, EVs have gained attention as a delivery vehicle for targeted delivery of oligo nucleotide drugs. Previous reports suggest that particles coated with targeting peptide can be delivered to selected cells and tissues. However, the biocompatible system for screening combinational libraries have not been described. The goal of this project is to develop a biocompatible system for peptide screening using EV-peptide display library.

Methods: Homologous recombination cloning using regenerative oligos are used to create peptide library plasmids where peptide sequences are fused to the phosphatidylserine binding domain (C1C2) of human lactadherin for peptide display to the vesicle surface. Fluorescent reporter gene was cloned with C1C2 domain for imaging purposes. Plasmid DNA was transfected to HEK293FT cells and EVs were harvested by ultracentrifugation followed by differential centrifugation. DNA sequence was recovered from EV by direct PCR amplification.

Results: Homologous recombination cloning was successfully used for EV library construction. Chimeric protein expression on EVs was determined by Western blot analysis and that of reporters was verified fluorescent microscopy. Direct detection of plasmid DNA was verified from isolated EVs and the targeting with known targeting peptide is in progress.

Summary/Conclusion: In this study, our current progress on developing *in vivo* peptide screening strategy using degenerative oligonucleotide library EVs will be discussed. The success of this approach may provide a novel biocompatible system for peptide screening both *in vitro* and *in vivo*.

Funding: Michigan State University Startup funding

PS01.09

The construction of nanogel/exosome hybrid by exosome surface polymer engineering

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Introduction: Extracellular vesicles secreted by various cells have attracted attention as a new system in cell-to-cell communication. We focus on the utilization of exosomes as biological molecule delivery systems. However, it is not always easy to control the delivery and internalization of exosomes to various cells. We propose here a new strategy for the effective delivery of exosomes into cells using functional macromolecular carriers such as amphiphilic nanogels. Surface polymer engineering was applied with a carrier of exosomes, namely, amphiphilic cationic CHP (cCHP) nanogel, to improve the delivery of exosome content by forming complexes with the exosomes. In this study, we developed the

preparation method of exosome hybrids with nanogel, and the hybrids were evaluated the characteristics and the biological functions.
Methods: Mouse macrophage cells were used to produce the exosomes, which were then mixed with cCHP nanogel to form a hybrid. Various characteristics of these hybrid particles were examined by TEM observation, nanoparticle tracking analysis to determine their size, measurements of their ζ-potential. The interactions between the hybrids and cells were evaluated by confocal scanning laser microscopy and flow cytometry.
Results: TEM revealed that the surface of each exosome was coated by cCHP nanogel particles. Flow cytometry also showed significant uptake of this exosome/nanogel hybrid by cells, with the main mechanism behind this internalization being endocytosis. A range of different molecules that inhibit different types of endocytosis were also applied to determine the particular pathway involved, with a caveola-mediated endocytosis inhibitor being revealed to markedly affect hybrid uptake. Next, we evaluated revealing the functional efficacy of this approach, we showed that the nanogel system could successfully deliver functional exosome into cells as indicated by its ability to induce neuron-like cell differentiation in the recipient cells.
Summary/Conclusion: These results indicate that the newly developed cationic nanogel systems for exosome delivery are powerful tools to investigate the biological functions of exosomes.

PS01.10

Human telomerized cells for production of extracellular vesicles

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Introduction: Human cells are of ever increasing importance as *in vitro* test system to represent the *in vivo* situation. Additionally, highly differentiated cells are also essential production systems for complex biopharmaceuticals. However, the use of such cell systems are limited due to the fact that the cells enter replicative life span and therefore can only be propagated for a limited number of population doublings *in vitro*, which limited standardization of experiments as well as production processes. Moreover, reports have shown that the number of secreted vesicles significantly reduced with increasing age of normal cells.
Methods: Human telomerase overexpression immortalizes cells while keeping primary like characteristics intact. Ectopic overexpression and charcterization of mesenchymal stem cells was used to establish production cell lines.
Results: Here we describe the development of human continuously growing cell lines from various tissues that show a high potential as innovative production systems for extracellular vesicles with use for clinical applications.
Summary/Conclusion: These cell lines will be used for the production of standardized EV preparation.

PS01.11=OWP1.06

Extracellular vesicles from Fat-laden hypoxic hepatocytes activates pro-fibrogenic signals in Hepatic Stellate Cells

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Introduction: Background: Transition from isolated steatosis (IS) to non-alcoholic steatohepatitis (NASH) is a key issue in non-alcoholic fatty liver disease (NAFLD). Recent observations in patients with obstructive sleep apnea syndrome (OSAS), suggest that hypoxia may contribute to disease progression mainly through activation of hypoxia inducible factor 1α (HIF-1α)-related pathways. Release of extracellular vesicles (EV) by injured hepatocytes may be involved in NAFLD progression.
Aim: To explore whether hypoxia modulates the release of EV from free fatty acid (FFA)-exposed hepatocytes and assess cellular crosstalk between hepatocytes and LX-2 cells (human hepatic stellate cell line).
Methods: HepG2 cells were treated with FFAs (250 μM palmitic acid + 500 μM oleic acid) and chemical hypoxia (CH) was induced with Cobalt (II) Chloride, which is an inducer of HIF-1α. Induction of CH was confirmed by Western blot (WB) of HIF-1α. EV isolation and quantification was performed by ultracentrifugation and nanoparticle tracking analysis respectively. EV characterization was performed by electron microscopy and WB of CD-81 marker. LX-2 cells were treated with 15 μg/ml of EV from hepatocytes obtained from different groups and markers of pro-fibrogenic signalling were determined by quantitative PCR (qPCR), WB and immunofluorescence (IF).
Results: FFA and CH-treatment of HepG2 cells increased gene expression of IL-1β and TGF-β1 in

HepG2 cells and increased the release of EV compared to non-treated HepG2 cells. Treatment of LX-2 cells with EV from FFA-treated hypoxic HepG2 cells increased gene expression of TGF-β1, CTGF, α-SMA and Collagen1A1 compared to LX-2 cells treated with EV from non-treated hepatocytes or LX-2 cells exposed to EV-free supernatant from FFA-treated hypoxic HepG2 cells. Moreover, EV from FFA-treated hypoxic HepG2 cells increased Collagen1A1 and α-SMA protein levels.

Summary/conclusion: CH promotes EV release from HepG2 cells. EV from hypoxic FFA-treated HepG2 cells evoke pro-fibrotic responses in LX-2 cells. Further genomic and proteomic characterization of EV released by steatotic cells under hypoxia are necessary to further delineate their role in the crosstalk between hepatocytes and stellate cells in the setting of NAFLD and OSAS.

Funding: FONDECYT 1150327-1150311.

PS02: EVs in Infectious Diseases and Vaccines II
Chairs: Norman Haughey; Ryosuke Kojima
Location: Level 3, Hall A

15:00–16:00

PS02.01

Host:pathogen interactions and host cell internalization of Trichomonas vaginalis exosomes

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Introduction: The parasite *Trichomonas vaginalis* is the causative pathogen of the sexually transmitted infection trichomoniasis. Depending on the parasite strain and host, infections can vary from asymptomatic to highly inflammatory. We previously reported that *T. vaginalis* generates and secretes vesicles with physical and biochemical properties similar to mammalian exosomes that deliver their contents to human host cells. *T. vaginalis* exosomes modulate host cell immune responses and likely assist in parasite colonization of the host.
Methods: In our current study, we are optimizing methods to study the uptake of *T. vaginalis* exosomes into the host cells.
Results: The data obtained from our studies show that exosome uptake is a time-dependent process, regulated by many factors such as temperature, etc. Our findings also suggest that exosome uptake is mediated by endocytosis, with specific host cell lipids playing a critical role in this process. We have also identified target molecules present on the surface of *T. vaginalis* exosomes that induce exosome uptake into the host cell.
Summary/Conclusion: This work expands our general knowledge of exosome uptake by target cells and our understanding of the mechanisms used by exosomes to mediate *T. vaginalis* host-pathogen interactions.
Funding: National Institutes of Health

PS02.02

Coating filter membranes with bacterial derived vesicles to study the permeation of anti-infectives across the Gram-negative cell envelope

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Introduction: Less and less novel anti-infectives against diseases caused by Gram-negative bacteria reach the market while bacterial resistance is steadily increasing. Among the many hurdles of an antibiotic on its way from development to clinical use, the Gram-negative cell envelope is one crucial factor strongly delimiting access to inner bacterial targets and thus decreasing efficacy. As a model to study and optimize the permeation of anti-infectives, outer membrane vesicles (OMV) were selected to create an *in vitro* membrane model on a 96-well filter plate.
Methods: E. coli BL21 were cultured in Luria-Bertani medium until stationary phase. Bacteria were separated by centrifugation (15 min, 9500*g*) and filtration (0.2 or 0.45 µm membrane pore size). OMV's were isolated by adding 33% (w/w) PEG 8000 solution to the filtrate (ratio 4:1), shaking and overnight incubation at 4°C. The precipitate was centrifuged (30 min, 16,233*g*) and the pellet resuspended in 100 µl filtered PBS. This suspension was characterized by nanoparticle tracking analysis and coated onto 96-well filter plates using a vacuum oven (15 min, 37°C, 100 mbar). Coating morphology was imaged by scanning electron microscopy and confocal laser scanning microscopy. For permeation studies the OMV coating was covered with 0.5% (w/v) agarose gel before adding solutions of different antibiotics to the donor compartment and determining the concentration time course in the acceptor compartment using UV-spectroscopy.
Results: The filtration through 0.2 and 0.45 µm pores led in both cases to sterile filtrates, whereas 0.45 µm pores led to larger vesicles and higher yield.

The applied microscopy methods indicated that a complete and homogenuous OMV coating was achieved. Preliminary permeation studies revealed kinetic differences between antibiotics.
Summary/Conclusion: The OMV isolation and purification protocol allowed for a yield sufficient to coat 96-well filter supports. The measured permeated amounts allow to distinguish the permeability of different antibiotics. Compared to artificial phospholipid membrane models, fluxes across OMV derived membranes were significantly higher, facilitating faster analytics. An

involvement of outer membrane proteins in this model is subject of ongoing investigations.

PS02.03

Quality markers for microbial EVs

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Introduction: Microbial EVs have potentially important roles in interactions with cells in populations of the same species, with other microbial species and with eukaryotic cells. To investigate the effect of these interactions in target cells it is important to define the EVs under test.

Methods: Pathogenic *Escherichia coli* 536 and 2348/69 and probiotic Nissle 1917 were cultured in RPMI 1640 ± FeCl3. Candida albicans and C. auris were cultured in YPD broth. Microbial EVs were separated from cells by centrifugation, filtration (0.2 µm for bacteria or 0.45 µm for yeast) followed by concentration (100,000 kDa cut-off filter) and ultracentrifugation. EVs were further enriched by either density gradient centrifugation (DGC, bacterial samples) or size exclusion chromatography (SEC, bacterial and yeast samples). An iTRAQ proteomic approach was used to identify proteins from bacterial cells, crude EV pellets and DGC and SEC fractions. Yeast proteins were fractionated by SDS/PAGE and proteins in EV-enriched and non-EV fractions were identified using mass spectrometry techniques.

Results: A number of outer membrane proteins were identified in *E. coli* EVs, but with some variation between strains and media used. Cytoplasmic protein GroEL was also common. There were no obvious proteins removed by the purification of EVs and the major differences in proteome were due to changes in environmental growth conditions. For Candida, a clear set of EV-associated envelope proteins were identified. In addition, a series of proteins removed from the crude EV prepartion by further enrichment were identified for Candida species that may represent non-EV contaminants.

Summary/Conclusion: A number of possible markers for *E. coli* and Candida species have been identified, which now need verification by alternative techniques and the screening of a range of pathogenic and non-pathogenic isolates grown in different conditions. These findings offer promising new markers for isolation of microbial EVs from both laboratory cultures and from clinical samples.

Funding: School of Medicine Performance-Based Research Fund; Maurice and Phyllis Paykel Trust Project Grant [8.1.17]; Lottery Health Research Grant [326702]; Health Research Council, Explorer Grant [14/805]; Ministry of Business, Innovation and Enterprise, Smart Ideas Grant [UOAX1507].

PS02.04

Akt and CD9 in urine exosomes as potential markers for urinary tract infection

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Introduction: Urinary tract infections (UTI) is one of the most common bacterial infections. UTI is treated with antibacterial agents, but asymptomatic bacteriuria (ABU) that is diagnosed by bacteriuria without any urinary tract symptoms should not be treated except pregnant women and patients who will undergo traumatic urologic interventions. However, there has been no clinically available biomarker to distinguish UTI from ABU. Exosomes are 40–150 nm sized membrane vesicles containing proteins and nucleic acids that are present within cells from which they are released and thus have the potential as biomarkers for various diseases. It is likely that urine may contain exosomes released from uroepithelial cells and white blood cells. In the present study, we aimed to identify urinary exosomal markers that are useful to discriminate between UTI and ABU.

Methods: Exosomes were collected by ultracentrifugation from the culture medium of SV-HUC-1 (immortalized uroepithelial cell line) and THP-1 (acute monocytic leukaemia cell line) co-cultured with or without *Escherichia coli* or treated with or without LPS. The protein expression was examined by western blot analysis. Urinary exosomes were isolated from urine by Tim4-conjugated magnetic beads. Expression of Akt and CD9 in isolated exosomes was analysed by ELISA and CLEIA, respectively.

Results: Expression of Akt, ERK and NF-κB was increased in exosomes isolated from SV-HUC-1 and THP-1 cells co-cultured with *E. coli* or treated with LPS compared to without co-culture or treatment. The

levels of Akt and CD9 in urinary exosomes from patients with UTI were higher than those from ABU patients.

Summary/Conclusion: Our results suggest that intracellular signalling molecule Akt and cell surface-resident exosomal marker CD9 in urinary exosomes have the potential to discriminate UTI from ABU, thus providing novel objective markers for their differential diagnosis, which will allow better diagnosis and treatment of UTI and ABU patients.

Funding: JSPS KAKENHI Grant Number JP18K09190, GSK Japan Research Grant 2015

PS02.05

Different protein profile and host immune response induced by extracellular vesicles from *Enterococcus faecium* cultured with or without antibiotics

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Introduction: Vancomycin-resistant *Enterococcus faecium* is of medical importance associated with multidrug resistance and opportunistic infection. *E. faecium* produces extracellular vesicles (EVs), but EV production in *E. faecium* under antibiotic stress condition and their pathogenic roles have not been determined yet. This study investigated the production of EVs in vancomycin-resistant *E. faecium* strain cultured with or without the sub-minimum inhibitory concentrations (MICs) of vancomycin and linezolid, and determined the pathogenic roles of EVs in colon epithelial Caco-2 cells.

Methods: The EVs were purified from vancomycin-resistant *E. faecium* ATCC 700221 cultured with or without the 1/2 sub-MICs of vancomycin and linezolid. Caco-2 cells were incubated with *E. faecium* EVs and then analysed for cytotoxicity and pro-inflammatory cytokine gene expression.

Results: *E. faecium* ATCC 700221 produced EVs during *in vitro* culture. *E. faecium* cultured with 1/2 sub-MIC of vancomycin and linezolid produced 4.5 and 2 times more EV proteins than bacteria cultured without antibiotics, respectively. A total of 438, 461 and 513 proteins were identified in the EVs isolated from *E. faecium* cultured in brain heart infusion (BHI) broth (EVs/BHI), EVs from *E. faecium* in BHI broth with 1/2 sub-MIC of vancomycin (EVs/VAN) and EVs from *E. faecium* in BHI broth with 1/2 sub-MIC of linezolid (EVs/LIN), respectively. EVs/BHI induced cytotoxicity and stimulated the expression of pro-inflammatory cytokine and chemokine genes in Caco-2 cells in a dose-dependent manner. Moreover, EVs/LIN were more cytotoxic towards Caco-2 cells than EVs/BHI and EVs/VAN, whereas EVs/VAN induced more pro-inflammatory cytokine and chemokine gene expression in Caco-2 cells than EVs/BHI and EVs/LIN.

Summary/Conclusion: The sublethal dose of antibiotics modulates the EV biogenesis in *E. faecium*. EVs produced by *E. faecium* under different antibiotic stress condition show different host cell responses, which plays a role in bacterial pathogenesis.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (NRF-2017R1A2A2A05001014).

PS02.06

The RNA profile of extracellular vesicles released from *Trypanosoma brucei*

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Introduction: Trypanosomes are unicellular eukaryotes, which are vector-borne ubiquitous parasites of vertebrates and have a high impact on global health. The well-known *Trypanosoma brucei* for instance is the causative agent of the human African trypanosomiasis, a lethal tropical disease, and the nagana-cattle disease in domestic livestocks in sub-Saharan Africa. Besides this, trypanosomes have become an important model organism, because of various biochemical and cellular characteristics such as *trans* spliced mRNAs. As other parasites, trypanosomes produce extracellular vesicles (EVs), which contribute to parasite-host interactions. Here we analysed for the first time the RNA profile from EVs produced by parasitic *T. brucei*.

Methods: We isolated EVs released from two different life cycle stages (procylic and bloodstream) of *T. brucei*, using a combination of differential centrifugation, size exclusion chromatography and ultracentrifugation. Subsequently we performed RNA-seq analysis of long RNAs (>200 nts) and small RNAs (<200 nts), followed by bioinformatic identification; validation of trypanosome and EV-associated RNAs was based on quantitative RT-PCR.

Results: Our analysis of RNAs revealed different RNA species in trypanosome-derived vesicles. Interestingly, we observed specific release of fragments from certain mRNAs into the vesicles, whereas metabolically important mRNAs were retained in the parasite, suggesting a role in RNA disposal. We are currently comparing the

mammalian- and insect-specific life cycle stages of the parasites, which should further clarify a potential functional role of vesicle-mediated host-parasite interactions.

Summary/Conclusion: Trypanosome-derived extracellular vesicles contain several RNA species, which are selectively released, representing a class of diagnostic biomarkers for diseases caused by these parasites.

Funding: LOEWE Center DRUID (Novel Drug Targets against Poverty-Related and Neglected Tropical Infectious Diseases).

PS02.07

Host immune response induced by outer membrane vesicles derived from *Burkholderia cepacia* cultured with different antibiotics

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Introduction: *Burkholderia cepacia* is an opportunistic pathogen that usually infects the patients with cystic fibrosis or indwelling hardware. This study investigated the production of outer membrane vesicles (OMVs) in *B. cepacia* strain cultured with the sub-minimal inhibitory concentrations (MICs) of antibiotics and their pathogenic roles *in vitro* and *in vivo*.

Methods: OMVs were purified from the culture supernatants of *B. cepacia* ATCC 25416 cultured with the 1/4 sub-MICs of ceftazidime (CAZ), trimethoprim/sulfamethoxazole (SXT) or meropenem (MEM). A549 cells were incubated with *B. cepacia* OMVs and then analysed for cytotoxicity and pro-inflammatory cytokine gene expression. Mice were treated with B. *cepacia* OMVs intratracheally, and lung pathology was evaluated.

Results: *B. cepacia* produced OMVs during *in vitro* culture. A total of 265 proteins were identified in OMVs isolated from *B. cepacia* cultured in Luria-Bertani broth (OMVs/LB) using proteomic analysis. OMVs/LB induced cytotoxicity and stimulated the expression of pro-inflammatory cytokine genes in lung epithelial A549 cells in a dose-dependent manner. *B. cepacia* produced more OMVs under antibiotic stress condition than under no antibiotic condition. Host cell cytotoxicity and pro-inflammatory response were significantly higher in A549 cells treated with OMVs from *B. cepacia* cultured with 1/4 sub-MIC of CAZ (OMVs/CAZ) than in the cells treated with OMVs/LB, OMVs from *B. cepacia* cultured with 1/4 sub-MIC of SXT (OMVs/SXT) or OMVs from *B. cepacia* cultured with 1/4 sub-MIC of MEM (OMVs/MEM). Intratracheal injection of OMVs/LB, OMVs/MEM, and OMVs/CAZ induced histopathology and pro-inflammatory responses in the mouse lung, but OMVs/SXT did not induce pro-inflammatory responses in the mouse lung. The expression of the interleukin-1β and GRO-α genes was significantly higher in the mice treated with OMVs/CAZ than the mice treated with other OMVs.

Summary/Conclusion: OMVs produced by *B. cepacia* exposed to different antibiotics represent different host cell responses, which may modulate influence on the bacterial pathogenesis.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (NRF-2017R1A2A2A05001014).

PS02.08

Thymol suppresses the inflammatory responses induced by *Staphylococcus aureus*-derived extracellular vesicles in cultured keratinocytes

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Introduction: *Staphylococcus aureus*-derived extracellular vesicles (EVs) deliver effector molecules to host cells and induce host cell pathology. This study investigated whether thymol could disrupt *S. aureus* EVs and suppress the pathology of the keratinocytes induced by *S. aureus* EVs.

Methods: Membrane disruption of the *S. aureus* EVs treated with thymol was determined using transmission electron microscopy. Human keratinocyte HaCaT cells were incubated with either intact or thymol-treated *S. aureus* EVs and then analysed for cytotoxicity and pro-inflammatory cytokine gene expression.

Results: Thymol inhibited the growth of *S. aureus* strains and disrupted the membranes of the *S. aureus* EVs. Thymol-treated *S. aureus* EVs inhibited the cytotoxicity of HaCaT cells when compared to intact *S. aureus* EVs; however, the cytoprotective activity differed between the EVs derived from *S. aureus* strains. Intact *S. aureus* EVs stimulated the expression of the pro-inflammatory cytokine and chemokine genes in keratinocytes. The expression levels of the cytokine genes differed between thymol-treated EVs from different *S. aureus* strains, but thymol-treated *S. aureus* EVs suppressed the expression of these genes. Thymol-

treated *S. aureus* EVs delivered lesser amounts of the EV component to host cells than intact EVs.

Summary/Conclusion: Our results suggest that the thymol-induced disruption of the *S. aureus* EVs inhibits the delivery of effector molecules to host cells, resulting in the suppression of cytotoxicity and inflammatory responses in keratinocytes. Thymol may attenuate the host cell pathology induced by an *S. aureus* infection via both the antimicrobial activity against the bacteria and the disruption of the secreted EVs.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (NRF-2017R1A2A2A0500 1014).

PS02.09= OWP2.09

Deciphering the role of extracellular vesicles on the blood–brain barrier during Zika virus infection

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Introduction: The association of Zika virus (ZIKV) with severe neurological disorders has gained increased interest over the last decade. However, the mechanism by which ZIKV crosses the blood–brain barrier (BBB) and reaches the brain remains to be elucidated. It is known that viruses incorporate viral material in extracellular vesicles (EVs) as a spreading strategy. These membrane-enclosed vesicles play a vital role in intercellular communication. Currently, there is a lack of knowledge on the possible involvement of EVs in ZIKV pathogenesis. Our study aims to unravel the role of EVs in ZIKV RNA transmission to the brain, via the BBB.

Methods: Human brain microvascular endothelial cells (HBMEC/D3) were used in our study since they represent the BBB *in vitro*. Three different EV isolation methods (precipitation kit, density gradient and size exclusion chromatography combined with the density gradient) were performed. Western blot, Transmission electron microscopy and Nanosight tracking analysis confirmed the presence of EVs in the supernatant of HBMEC/D3 cells. The presence of ZIKV RNA in infected-EVs (IEVs) was evaluated by immunofluorescence and qPCR. In addition, the effect of IEVs on the BBB was assessed using a label-free impedance-based biosensor (ECIS, Applied BioPhysics).

Results: We confirmed the presence of viral components in our IEVs, including the NS1 and E proteins of ZIKV. The obtained IEVs were able to re-infect susceptible cells, even after being pretreated with RNase A. This indicates that the viral RNA resides inside the IEVs. Using impedance measurements on HBMEC/D3 cell monolayers, we observed that IEVs, as well as virus control caused similar and temporal disturbances on the monolayer's integrity within 30 min post infection. No disturbances were seen upon addition of non-infected EVs.

Summary/conclusion: Our study demonstrates that EVs-derived from ZIKV-infected cells are able to transfer proteins and viral RNA to recipient cells. Since both IEVs and viral particles can induce similar changes on barrier's integrity it is possible that IEVs are involved in an alternative mechanism of ZIKV transmission.

PS02.10=OWP2.11

In vivo testing of OMV-based vaccine prototypes against *Gallibacterium anatis*

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Introduction: Outer membrane vesicles (OMVs) are produced by the majority of Gram-negative bacteria. Thanks to the antigenic similarity between OMVs and the bacterial outer membrane, OMVs have proven to be promising for the development of novel vaccines against bacterial pathogens. In this work, we describe the testing of OMV-based vaccine prototypes against *Gallibacterium anatis*, a Gram-negative pathogen of great veterinary interest.

Methods: OMVs were isolated from a *G. anatis* hypervesiculating mutant using a modified version of the Hydrostatic Filtration protocol described by Musante et al. (2014). 120 16-week-old Lohmann-Brown chickens were divided in six groups and immunized twice intramuscularly with different combinations of buffer (controls), OMVs and selected recombinant immunogens. Two weeks after second immunization, the effectiveness of the immunization regimes adopted was tested by challenging the animals intraperitoneally with live CFUs from a heterologous *G. anatis* strain. One week post-challenge, the animals were sacrificed and an established lesion score model was used during necropsy to evaluate the clinical outcome of infection.

Results: Statistical analysis of the recorded lesion scores showed that the group immunized with *G. anatis* OMVs presented an average total score of 2.95, as opposed to an average total score of 8.77 in the control group. The approximately threefold reduction in total

average lesion score observed demonstrates that immunization with *G. anatis* OMVs is able to effectively decrease the morbidity of *G. anatis* infection in the immunized animals.

Summary/conclusion: Our results show that *G. anatis* OMVs represent a promising candidate for the development of cost-effective vaccination strategies for the prevention of *G. anatis* infections in a cross-serovar manner. Accordingly, we hypothesize that dose/ response optimization and the enrichment of *G. anatis* OMVs with selected immunogens should result in an improvement of the effectiveness of the vaccination regime proposed.

Funding: This research project is being funded by a grant from Huvepharma (https://www.huvepharma.com/).

PS03: EVs in Cardiovascular Disease
Chairs: Oh Youn Kim; Caroline Reddel
Location: Level 3, Hall A

15:00–16:00

PS03.01

Serum exosome mediates chronic intermittent hypoxia-associated endothelial dysfunction through regulating miR-144, −27a/Nrf2 pathway

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Introduction: Endothelial dysfunction plays a crucial role in the development of OSAHS-related vasculopathy, but the mechanisms are not fully understood. Exosomes, abundant in blood, deliver various molecules to recipient cells. However, whether serum exosomes (SExos) are involved in chronic intermittent hypoxia (CIH)-associated vasculopathy is largely unknown.

Methods: SExo was purified by ultracentrifugation. TEM and NTS were used to evaluate the purity of exosome. Myograph was used to detect endothelial function. Western blotting and quantitative polymerase chain reaction (qPCR) were used to measure the expression of protein or miRNA respectively. Confocal microscopy was used to detect the signal of exosome or oxygen free radical in endothelial cells.

Results: Endothelial dysfunction caused by CIH was related with oxidative stress. Furthermore, SExos from CIH mouse (CIH SExos) severely impaired endothelial function and enhanced the oxygen free radical in endothelial cells from normal C57BL/6 mice. Western blotting showed that the expression of antioxidant transcription factor Nrf2 and its downstream target catalase were significantly decreased in CIH SExos-treated endothelial cells *ex vivo* or *in vitro*. qPCR assay showed significant increasement of exosomal miR-27a and miR-144 under CIH status. Correspondingly, silencing miR-144 and miR-27a with CIH SExo-packaged antagomiR-27a and antagomiR-144 confirmed the pivotal role of SExo miR-27a and miR-144 in CIH SExo-inhibited Nrf2 expression, CIH SExo-induced endothelial dysfunction and the excess oxygen free radical generation in endothelial cells.

Summary/Conclusion: This study demonstrates the adverse effect of CIH SExo on endothelial cells, representing a novel cellular mechanism of exosomal miR-27a, −144/Nrf2 pathway that mediates the development of CIH-associated endothelial dysfunction.

Funding: This study was supported by National Natural Science Foundation of China (No.81471082, 31741064 and 81470567)

PS03.02

Influence of cardiovascular risk markers on numbers and characterization of circulating extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are small plasma membrane-derived vesicles released from various cells, which potentially affect many pathophysiological processes involved in cardiovascular diseases (CVDs). However, there is little information about the relationship between gender, CVD risk markers (Body Mass Index (BMI), blood pressure (BP), triglyceride level, cholesterol level and HDL level), CVD risk score and circulating EVs.

Methods: Subjects (n = 27) aged 40–70 years with moderate risk of CVDs (QRISK2 score) were recruited and assessed for BMI, BP and blood lipid profile. EVs were isolated from platelet-free plasma by size exclusion chromatography and analysed by nanoparticle tracking analysis (NTA) and flow cytometry (FCM). NTA measured the concentration and size distribution of EVs, and EVs were phenotyped by FCM via a 3-colour panel, including Annexin V (for the majority of circulating EVs), CD41 (for platelet-derived EVs) and CD105 (for endothelial-derived EVs).

Results: • Subjects unexpectedly fell into two clear groups: high EVs group (total EV numbers: 4*10^10/mL blood ~ 8*10^10/mL blood, n = 9 or Annexin V + EV numbers: 2.6*10^7/mL blood ~ 5*10^7/mL blood, n = 17) and low EVs group (total EV numbers: 1*10^10/mL blood ~ 3.9*10^10/mL blood, n = 18 or Annexin V+ EV numbers: 9*10^6/mL blood ~ 2.5*10^7/mL blood, n = 10).

• Males accounted for 78% of the subjects in high total EVs group. Overweight subjects (BMI ≥ 24.9 kg/m^2) contributed to 89% of the subjects with high total EV

numbers, while 93% of the subjects with normal weight were classified into low EVs group. The high Annexin V+ EVs group had significantly higher diastolic BP levels (p = 0.02) and higher cholesterol levels (p = 0.03) than those with low EV numbers. Those with higher total EV numbers had a higher average CVD risk score (p = 0.02).
• Overweight subjects had a significantly higher number of endothelial-derived EVs than subjects with normal weight (p = 0.02).
Summary/Conclusion: The majority of subjects with high total EV numbers were male. Overweight contributed to the elevation of total EV and endothelial-derived EV numbers. Higher BP level, cholesterol level and CVD risk score were associated with higher numbers of circulating EVs.
Funding: This project is supported by Biotechnology and Biological Sciences Research Council (BBSRC)–Diet and Health Research Industry Club in UK

PS03.03

Changes in exosome release in ageing: a pilot study in a human model of ischemia reperfusion

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Introduction: The growing aged population necessitates better understanding of cellular and physiological changes in ageing to improve future healthcare delivery and cost. The role of exosomes, extracellular vesicles carrying biologically active cargo secreted by almost all cells, may have major impacts on perioperative care and monitoring. Deep hypothermic circulatory arrest (DHCA) is a profound perioperative stress event involving hypothermia, arrest of circulation to major organ systems and whole body ischaemia reperfusion. DHCA is used during pulmonary thromboendarterectomy, for which the University of California, San Diego, USA, serves as a leading centre. With a patient age range of 14–80 years old, we use DHCA as a model of whole body ischaemia reperfusion to test the novel hypothesis that DHCA alters the amount of exosome release, content and ability of exosomes to affect cellular metabolism and function in an age-dependent manner.
Methods: Plasma was obtained from patients undergoing DHCA: after induction of anaesthesia (baseline), at initiation of cardiopulmonary bypass (CPB), completion of cooling, after circulatory arrests and at chest closure. Exosomes were isolated with ExoQuick. Nanoparticle tracking analysis (NTA) measured particle concentration. Immunoblotting and electron microscopy confirm the presence of exosomes. Samples were stored for proteomic, microRNA and *in vitro* analysis.
Results: Mean particle sizes at each time point were within the known size distribution of exosomes. Particle concentration at the completion of cooling was decreased from baseline. Thereafter, particle concentration showed an increase after DHCA and a further increase during chest closure at the conclusion of the surgery.
Summary/Conclusion: Our data show that cooling can decrease exosome levels in blood, while whole body ischaemia reperfusion associated with DHCA in patients may be a stimulus for exosome release. As more samples are collected, we will assess changes in the proteome and microRNA content of exosomes before and after DHCA as a function of age. This model also lends itself well to further detailed investigation of tissue and organ-specific responses to ischaemia reperfusion in young and aged patients.
Funding: This work was funded by the National Institutes of Health, USA.

PS03.04

Intracardiac extracellular vesicle release in post-infarction diabetic hearts

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Introduction: Cardiovascular disease (CVD) is the main cause of death in non-communicable diseases. In response to myocardial infarction (MI), extracellular vesicles (EVs), including large (lEVs) and small (sEVs), are released within and from the heart to facilitate intercellular communication and maintain cardiac homeostasis. As diabetes increases the risk of CVD, the purpose of the study was to investigate how diabetes influences the release of intracardiac EVs after MI.
Methods: C57BL/6J male mice were fed normal chow diet or high-fat diet (HFD) for 3 months. HFD-fed mice were glucose intolerant as attested by the measure of GTT above 200 mg/mL. Mice were then subjected to MI by permanent ligation of the left anterior descending artery, and sham animals underwent similar surgical procedure without ligation. Left ventricles from sham or MI mice were then harvested at either 15, 24, 48 or 72 h after surgery (n = 5 per group at each

time point) and processed for EV extraction by differential centrifugation. lEVs and sEVs were then quantified and analysed via Tunable Resistive Pulse Sensing Technology (TRPS), flow cytometry and Western blot.
Results: In chow diet-fed mice, release of both lEVs and sEVs was increased at 24 h post-surgery when compared to shams. These findings were in agreement with previous data obtained in younger control animals. In diabetic mice, lEVs peaked at 24 h post-MI and this increase was slightly greater than that observed in chow diet-fed animals. However, there were no differences in sEV release between sham and MI diabetic mice. TRPS analysis revealed that diabetes does not change EV size (diameter) and population. Furthermore, both control and diabetic-derived EVs harboured cardiomyocyte marker (Troponin T) as revealed by Western blot.
Summary/Conclusion: Our results thus show that diabetes modulates the release of both large and small intracardiac EVs after MI. Further work will be needed to fully investigate the functional impact of cardiac EVs in the diabetic heart after MI.
Funding: INSERM and ANR-16-CE92-0032-02

PS03.05

Exosomal low-density lipoprotein receptor (LDLR) as a potential biomarker in patients with coronary artery disease

Dapi Meng Lin. Chiang[a], Liv Weichien Chen[b], Michael Pfaffl[c] and Chin-Sheng Lin[b]

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Introduction: Atherosclerosis is one of the key factors contributing to cardiovascular disease. Exosomes have been documented to be associated with atherosclerosis pathogenesis. However, the potential exosome-related biomarkers in atherosclerosis patients has not been analysed and characterized yet. In this study, we aimed for assessing the potential biomarker in serum exosome for coronary artery disease (CAD).
Methods: Plasma samples were collected from patients undergoing coronary angiography. To assess exosomal low-density lipoprotein receptor (LDLR) and ATP-binding cassette transporter A1 (ABCA1) expression, we isolated exosome by incubating glycan recognition beads, EXÖBead (Biovesicle) with 250 μL pre-cleared plasma from healthy donors (n = 28) and CAD patients (CADs, n = 26) as manufacturer's protocol. To confirm the purity of our isolation, we used NTA and TEM to demonstrate exosome morphology and size distribution, according to the MISEV guidelines. Exosomal LDLR and ABCA1 protein expressions were analysed by flow cytometry, FACS. Furthermore, the exosome specific markers CD9, CD63 and CD81 were simultaneously detected in exosome-EXÖBead complexes by multiple fluorescent antibody staining and FACS. We incorporated 10% exosome-free "foetal bovine serum" in PBS as the antibody staining negative control.
Results: The exosome size distribution and morphology were similar between the plasma sample from healthy and CAD groups. The geometric mean fluorescence intensity, MFI of CD9, CD63, CD81, LDLR and ABCA1 were not different between these two groups. However, the corrected MFI ratio of LDLR/CD9 in healthy donors was significantly higher compared to CAD patients (p = 0.044). Similar significant changes in ratio of LDLR/CD63 (p = 0.026) and LDLR/CD81 (p = 0.027) were also observed. Besides, there is no significant change in exosomal ABCA1 between healthy donors and CAD patients.
Summary/Conclusion: Declined expressions of LDLR/exosome in patients with CAD were observed in our study. These results may be an essential clue for exploring the function of exosomal LDLR in lipid metabolism and atherosclerosis. Further approaches regarding cell-to-cell communication of exosomal LDLR will be addressed in the future.

PS03.06

Therapeutic EV rescue a deficient hypoxic response in pulmonary arterial hypertension

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Introduction: Complex gene-environment interactions can determine the penetrance of a genetic mutation leading to disease. Pulmonary arterial hypertension (PAH) is a lethal disease that is highly associated with loss-of-function bone morphogenetic protein receptor II (BMPR2) mutations and persistent hypoxic stress. While these genetic and environmental determinants of PAH are clearly defined, little is known about how they are interconnected to potentiate disease.
Methods: Pulmonary arterial endothelial cells (PAEC) were exposed to hypoxia (0.5% O2) for 24 h and culture media collected. Extracellular vesicles (EVs) were isolated using differential ultracentrifugation and characterized with electron microscopy and nanoparticle analysis. qPCR analysis after RNAse digestion was performed to identify packaged mRNA. EV treatment

of mice was administered via tail-vein injection. *In vivo* biodistribution was visualized by Gallium-68 labelling coupled with positron emission tomography (PET) imaging.
Results: Here we report that BMPR2 is induced by hypoxia in PAEC, and loss of BMPR2 partially blocks the release of extracellular vesicles under these conditions. EVs derived from hypoxic PAEC are enriched with BMPR2 mRNA and can restore abnormal phenotypes in BMPR2 mutant cells *in vitro*. BMPR2 knockout mice exposed to intermittent hypoxia develop PAH phenotypes that are prevented by treatment with hypoxic PAEC derived EV.
Summary/Conclusion: These results show that PAEC-derived EVs are critical for the maintenance of vascular homeostasis, loss of this signal due to BMPR2 dysfunction contributes to PAH pathogenesis, and replacement with exogenous EV has therapeutic potential in PAH.
Funding: F32 HL 132452, NIH NHLBI

PS03.07

Role of extracellular vesicles in cardiovascular toxicity induced by BCR-ABL tyrosine kinase inhibitors

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Introduction: Despite their efficacy as an anti-cancer therapeutic against chronic myelogenous leukaemia (CML), tyrosine kinase inhibitors (TKIs) can be associated with deleterious cardiovascular effects. Considerable progress has been made in identifying the excess risk of cardiovascular events related to TKI exposure; however, the data on the underlying mechanisms and possible predictive biomarkers are currently inadequate. To this end, we sought to examine EV-associated miRNAs as a means of elucidating their potential as effectors and biomarkers of TKI-induced cardiovascular toxicity in CML.
Methods: We obtained informed consent and recruited 24 age- and sex-matched response stable CML patients either off-TKI (median 32.26 months, $n = 6$) or on long-term treatment with imatinib, nilotinib or ponatinib (median 79.01 months, $n = 6$/group), and assayed plasma-derived EV-associated miRNAs using the nCounter® Analysis System. Concurrently, *in vitro* studies were conducted to examine the responses of iPSC-derived human cardiomyocytes to plasma-derived EVs using BNP as a surrogate marker of the cardiovascular stress response. Consent was obtained by the University Health Network Research Ethics Board.
Results: We identified selective dysregulation in each treatment group associated with CML and as well as specific cardiovascular pathophysiology, e.g. miR-let-7e-5p, miR-502-5p and miR-548a-5p ($p < 0.05$). *In vitro*, we identified a surprising cardioprotective action of ponatinib-patient EVs on cardiomyocytes, indicated by a significant decrease in free BNP in the media of cardiomyocytes treated with EVs compared to other treatment arms ($p < 0.01$).
Summary/Conclusion: This study represents a novel approach investigating the utility of EVs and their associated miRNAs as biomarkers and effectors of TKI-induced cardiovascular toxicity. Our results highlight a distinct profile of miRNAs associated with TKI treatment. Understanding the complex role of EVs in TKI therapy will elucidate the complexities of cardiovascular toxicity and aid in tailoring the risk management of individual patients.
Funding: This project was funded by the Princess Margaret Cancer Centre.

PS03.08

Extracellular vesicles derived from genetically modified human induced pluripotent stem cells enhance cardiomyogenesis and angiogenesis *in vitro* and *in vivo*

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Introduction: Extracellular vesicles (EVs) represent population of small circular membrane vesicles secreted by most cells including stem cells (SCs). It has been reported that EVs may carry bioactive cargo including proteins, microRNAs and mRNAs. They also play a crucial role in cell-to-cell communication in both physiological and pathological conditions.

The aim of this study was to verify the impact of EVs derived from human induced pluripotent stem (iPS) cells (hiPS-EVs) overexpressing procardiomyogenic miR1 or miR199a, or proangiogenic miR126, on various properties of human cardiac and endothelial cells.
Methods: hiPS-EVs were isolated from conditioned hiPS culture media by differential centrifugation including ultracentifugation. Cardiac cells and endothelial cells were used as target cells *in vitro*, and their functional properties were evaluated after hiPS-EVs treatment. The regenerative capacity of hiPS-EVs

was also examined *in vivo* – in murine model of acute limb ischaemia (LI).

Results: Our data indicate that hiPS-EVs carrying pro-cardio- and proangiogenic miRNAs may protect cardiac cell types from apoptosis as well as enhance their proliferation, metabolic activity, migration and cardiomyogenic differentiation. The hiPS-EVs enhanced also proangiogenic capacity, migration and metabolic activity of HCAEC cells *in vitro*. The vesicles also promoted angiogenesis and increased blood flow recovery in murine ischaemic limb injury model *in vivo*.

Summary/Conclusion: These results may indicate (i) feasibility of genetic modifications of EVs enforcing their regenerative proprieties as well as (ii) enhanced activity of EVs from hiPS cells overexpressing miR1, miR199a and miR126 in regeneration of ischaemic tissues. We conclude that EVs from genetically modified hiPS cells may represent new safe tool for tissue repair alternative to whole-cell therapies *in vivo*.

Funding: This study was funded by NCN and NCBR grants: SONATA BIS-3 (UMO-2013/10/E/NZ3/007500) and STRATEGMED III (STRATEGMED3/303570/7/NCBR/2017) to EZS and PRELUDIUM-11 (UMO-2016/21/N/NZ3/00363) to KKW.

PS03.09

Cardioprotective and proangiogenic potential of small extracellular vesicles secreted from amniotic fluid stem cells

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Introduction: Mesenchymal stem cells (MSCs) exhibit antiapoptotic and proangiogenic functions in models of myocardial infarction, a common cause of death and disability. These effects are partially mediated by secreted small extracellular vesicles (sEVs). Amniotic fluid stem cells (AFSCs) are foetal MSCs with superior functional potential to adult MSCs. We hypothesized that sEVs released by AFSCs are cardioprotective and proangiogenic.

Methods: Human AFSC sEVs were isolated from serum-free conditioned medium by size-exclusion chromatography and characterized using nanoparticle tracking, dot blots, protein and immunoassays, electron microscopy and protein arrays. Their cardioprotective potential was examined in models of hypoxia/reoxygenation- and reactive oxygen species-induced death of primary adult rat cardiomyocytes *in vitro*. AFSC sEV effects on human endothelial cell migration, proliferation and signalling pathway activation were also investigated (using Boyden's Chamber assay, MTT assay and western blot analysis/phosphokinase arrays, respectively).

Results: Isolated AFSC sEVs were CD9/CD63/CD81-positive and of high purity (up to 1.2x10^10 particles/μg protein). These vesicles were not cardioprotective in models of simulated ischaemia/reperfusion injury in primary cardiomyocytes *in vitro*. Nevertheless, AFSC sEVs carried promigratory cytokines and angiogenic factors (e.g. SDF-1, MIF, PTX3) and promoted endothelial cell migration and proliferation *in vitro*. Pharmacological inhibition of PI3K (a promigratory signalling pathway) in target endothelial cells reduced sEV-stimulated migration by 54 ± 15% ($p < 0.001$). However, sEVs did not induce phosphorylation of downstream PI3K targets, indicating that sEV effects may be multifactorial and may involve multiple pathways.

Summary/Conclusion: AFSC sEVs did not have direct protective effects on cardiomyocytes *in vitro* but possessed proangiogenic potential which requires, but is not solely dependent on, PI3K signalling. Ongoing experiments include analyses of the sEV proteome, their cardioprotective properties in a model of rat myocardial ischaemia/reperfusion injury *in vivo* and their role in capillary sprouting from rat aortic explants. Together, these data will define the potential for using AFSC sEVs as cardioprotective and proangiogenic therapy.

Funding: BHF

PS03.10

CystatinC and CD14 in plasma extracellular vesicles are associated with both renal dysfunction and heart failure in patients presenting with dyspnoea

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Introduction: Heart failure and renal failure commonly coexist: heart failure patients have higher chance of developing renal failure and vice versa.[1,2] Declines in renal function are associated with the development of ventricular dysfunction and worsen prognosis in heart failure.[3,4] However, underlying pathophysiological mechanisms in cardiac-renal cross talk are not fully understood[1]. The role of plasma extracellular vesicles (EVs) in combined organ failure such as cardiorenal syndrome has not been investigated. The primary aim

of this study is to investigate if the extracellular vesicle proteins CystatinC, CD14, SerpinG1 and SerpinF2 that have been associated with heart failure are also associated with renal dysfunction in patients with acute dyspnoea.

Methods: Blood samples were prospectively collected in 404 patients presenting with breathlessness at the emergency department at National University Hospital, Singapore. Renal dysfunction was defined as estimated glomerular filtration rate below 60 mL/min/1.73m2. The presence of heart failure was independently adjudicated by two clinicians. EVs were precipitated in 3 sub-fractions from the plasma using dextrane sulphate for the LDL and HDL plasma-subfractions or ExoQuick. After precipitation, the EVs were lysed and the four selected proteins were measured quantitatively using immunobead assays and tested for their associations with renal dysfunction, heart failure and the concurrence of both conditions using multinomial regression analysis.

Results: CystatinC was associated with renal dysfunction, heart failure and their combination in all three EV-sub-fractions and in plasma. CD14 was associated with both renal dysfunction and the combined renal dysfunction and heart failure in all EV-sub-fractions, and with the presence of heart failure in the HDL-subfraction but these associations were only seen in the EV subfractions and not in plasma.

Summary/Conclusion: We provide the first data showing that EV CystatinC and CD14 are associated with both renal dysfunction and heart failure in patients presenting with acute dyspnoea. These data suggest that extracellular vesicle proteins may be involved in the combined organ failure of the cardiorenal syndrome, and represent possible targets for prevention or treatment.

PS03.11=OWP1.03

Identification of extracellular vesicles as biomarkers for myocardial infraction by flow cytometry and automated data processing

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Introduction: Acute myocardial infarction (AMI) is a major cause of death. To diagnose AMI, measuring troponin concentration is the gold standard. Since troponin is unspecific for AMI, novel biomarkers for AMI are urgently needed. After the onset of AMI, platelets, endothelial cells and blood cells release specific extracellular vesicles (EVs). Our aim is to identify these EVs as biomarkers for AMI diagnosis and treatment monitoring.

Methods: The study was approved by the medical ethics committee. Venous blood was collected 24 hours, 72 hours and 6 months after AMI from fasting patients (n=60, 64.5±10.8 years, 68% male) and healthy controls (n=30, 57.7±6.6 years, 62% male). Flow cytometry (Apogee A60 Micro) was used to determine plasma concentrations of EVs labelled with antibodies for activated platelets (CD61, CD62p; PEVs), endothelial cells (CD146; EEVs) and red blood cells (CD235a; RBC-EVs). Processing of 1,224 flow cytometry data files was performed using in-house developed, automated software (MATLAB R2018a), enabling flow rate stabilization, diameter and refractive index determination, MESF calibration, fluorescent gate determination and statistics reporting.

Results: Between AMI patients and controls, PEV concentrations in plasma were comparable (p=ns), EEV concentrations increased ($p<0.0001$), and RBC-EV concentrations decreased ($p<0.0001$). Antiplatelet drug ticagrelor decreased concentrations of PEVs ($p=0.03$), compared to less potent clopidogrel, but did not affect EEVs and RBC-EVs. In turn, concentrations of EEVs, but not PEVs and RBC-EVs, positively correlated with the dose of atorvastatin ($p<0.001$). The antioxidative β-blocker carvedilol increased concentrations of RBC-EVs, compared to nebivolol ($p=0.05$), but did not affect PEVs and EEVs.

Summary/Conclusion: Flow cytometry and automated data processing were used to find biomarkers for AMI based on EVs in plasma. During treatment, ticagrelor decreased PEV concentrations, atorvastatin increased EEV concentrations, and carvedilol increased RBC-EV concentrations, suggesting that EVs might be used to monitor AMI treatment. AMI patients differed from controls regarding EEV and RBC-EV concentrations, but not PEVs, likely because blood was collected 24 hours after the start of antiplatelet therapy. In follow-up studies, it is crucial to collect blood prior to treatment.

PS04: Affinity and Microfluidic Separation
Chairs: Kazunari Akiyoshi; Yanling Cai
Location: Level 3, Hall A

15:00–16:00

PS04.01

Isolation of extracellular vesicles from small volume of plasma by microfluidic aqueous two phase system

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Introduction: Isolation of extracellular vesicles (EVs) from small volume of sample is a major issue of point-of-care testing and it leads to great attention in microfluidic device. However, previous microfluidic immunoaffinity approach has possibility of the loss of EVs that might have more useful information due to heterogeneity of EVs. In the case of microfluidic device applying external forces, has drawback in complicated fabrication process and possibility in deformation of EVs. Therefore, this paper suggests a microfluidic aqueous two phase system (ATPS) in isolation of EVs from stable laminar two phase flow with just simple design of chip.
Methods: EV-protein mixture was tested to investigate the partitioning behaviour. EVs were isolated by ultracentrifuge from human plasma, then bovine serum albumin was added to prepare EV-protein mixture. Polyethylene glycol (PEG, 3.5 wt%) dissolved in phosphate-buffered saline was injected to top and bottom inlet. Dextran (DEX, 1.5 wt%) dissolved in sample was injected to middle inlet. Fluorescence intensities of EV and albumin were imaged to investigate the partitioning behaviour in real time from EV-protein mixture. Concentrations of collected EV and albumin were measured to confirm the fluorescence imaging. Also, same experiment was performed with only PEG without dextran to investigate the effect of ATPS. EV isolation from human plasma was also performed and characterized by western blot and atomic force microscopy.
Results: Most of green EVs were remained in middle phase where red BSA seems almost fully diffused out for the equilibrium state in fluorescence experiment. Microfluidic ATPS could isolate the EV with 83.43% of recovery efficiency and protein removal of 65.46% from EV-protein mixture. Microfluidic without ATPS could isolate the EV with recovery rate of 67.14%. Also, EVs were successfully isolated from human plasma with almost same recovery rate.
Summary/Conclusion: The difference of diffusion velocity in laminar flow was dominant factor in separating proteins from EVs in our microfluidic ATPS. Other body fluids will be tested with our modified system. We expect that our device will provide more useful application in isolation of EVs.

PS04.02

Extracellular vesicle-associated microRNAs show stronger correlations with cardiovascular disease protein biomarkers than cell-free microRNAs in human plasma

Shi Chen[a], Shu-Chu Shiesh[b], Gwo-Bin Lee[c] and Chihchen Chen[a]

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Introduction: This abstract presents a high-efficiency method utilizing two sets of magnetic beads to isolate extracellular vesicles (EVs) and EV-associated microRNAs (EV-miRNAs) from human platelet-poor plasma samples. Our goal is to develop a platform for risk assessment of cardiovascular diseases (CVDs) and compare the expression levels of circulating cell-free miRNAs and EV-miRNAs. In contrast to the rapid peaking and falling of cardiac troponin I (cTN-I), a conventional CVD biomarker, the level of circulating miR-126 remains downregulated even one week after the onset of acute myocardial infarction (AMI).
Methods: In this study, we first used anti-CD63 antibody-coated magnetic beads to separate CD63+ EVs. EV-miRNAs were released after EV lysis and subsequently extracted by using oligonucleotide-conjugated magnetic beads. Expression levels of cell-free and EV-associated microRNAs in six clinical plasma samples were quantified using quantitative reverse transcription polymerase chain reaction (RT-qPCR) with a spike-in exogenous cel-miR-238 control.
Results: Experimental results showed the levels of miRNAs in CD63+ EVs were ~74% of cell-free miRNAs in plasma, whereas the miRNA extraction

efficiency was >87% and exhibited no apparent dependence on the concentration of miRNA and the medium evaluated. Compared with the levels of conventional CVD protein biomarkers, EV-derived miR-126 levels were negatively correlated with N-terminal pro-b-type natriuretic peptide (NTproBNP) and cTN-I levels with $R^2 = 0.70$ and $R^2 = 0.61$, respectively. In contrast, circulating total miR-126 levels were only weakly correlated with these biomarkers ($R^2 = 0.14$, $R^2 = 0.02$, respectively).

Summary/Conclusion: We have developed methods to isolate EVs from human plasma samples, and subsequently to extract miRNAs carried by EVs by using two sets of magnetic beads. Our preliminary results suggest that EV-associated miR-126 may serve as a better biomarker than the total circulating miR-126. More clinical samples are currently being investigated.

Funding: Taiwan Ministry of Science & Technology (MOST 106–2221-E-007–003-, MOST 105–2221-E-007–009-, and MOST 106–2221-E-007–002-) and the Taiwan Ministry of Education (Higher Education Sprout Project: grant no. 107Q2713E1).

PS04.03

Effective separation of exosomes based on its surface sugar chains using a macroporous spongy monolith

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Kyoto University, Kyoto, Japan

Introduction: The surface sugar chains on exosomes contribute the communication among cells. But, in the present separation procedures, the effective separations of exosomes based on the differences of sugar chains have never reported. We focus on a lectin affinity chromatography (LAC) with a macroporous spongy monolith (1), which is suitable for a high throughput and selective separations for biomolecules. In this study, we prepared a few lectin-immobilized spongy-monolithic columns and evaluated for typical LAC analyses. Additionally, the columns were applied for the separation of exomes to determine the fundamental adsorption/desorption conditions.

Methods: Poly(ethylene-co-glycidylmethacrylate) (PE GM)-based spongy monolith (PEGM-SPM) was packed into columns, and then concanavalin A (ConA) or *Sambucus sieboldiana* agglutinin (SSA) was immobilized. Additionally, bovine serum albumin or insulin (Ins) was further immobilized to block the hydrophobic surface of PEGM-SPM. The obtained columns were simply analysed by LAC and applied for the separation of exosomes.

Results: As results of LAC evaluations, both ConA-SPM and SSA-SPM showed selective lectin affinity for the glycoproteins, only the glycoproteins associated to each lectin were selectively separated from the mixture samples. Additionally, an Ins-SPM allowed the effective permeability against liposome and exosome. This means that the protein-immobilized SPM was suitable for the separation media of nanometer sized particles without any non-specific adsorption. Finally, we demonstrated the selective separation of exosome due to lectin affinity. As a result, SSA-SPM provided the effective adsorption of exosome based on the interaction between SSA and sialic acid on exosome.

Summary/Conclusion: According to these results, the newly developed lectin-SPMs can be used for the separation of exosomes based on the difference of the surface sugar chains. We believe that the increase of number of lectin-SPMs and other affinity-SPMs will lead to the detailed classification of exosomes due to its surface chemistry.

(1) Kubota, K.; Kubo, T.; Tanigawa, T; Naito, T.; Otsuka, K. Sci. Rep. 2017, 7, 178.

PS04.04

A microfluidic module for extracellular vesicle separation coupled to microarray-based phenotyping

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Introduction: Standard approaches to characterize EVs are usually either low-throughput, laborious or based on sophisticated equipment not applicable to clinical routines. An integrated microfluidic platform that isolates and characterizes EVs available in bodily fluids by combining capture, release and phenotyping of bio-nanoparticles would significantly accelerate the transition of EV-based research to real clinical utility. The realization of such a system is the goal of INDEX, a project recently funded in the frame of Horizon 2020 FET-OPEN programs.

Methods: The platform consists of two modules, one for the extraction of EVs in complex samples and one for the interferometric label-free identification and visualization in a disposable cartridge. In order to integrate the two modules, a new approach for the reversible capture of EVs from serum was developed and demonstrated using the so called magnetic fluidized bed technology recently developed by Pereiro et

al. (Lab Chip, 2017, 17, 1603–1615). This technology is based on the use of functionalized magnetic particles to perform a solid phase extraction step. We report on the progress of the optimization of the technology itself and how it can be used to recover intact EVs.

Results: In the optimization of the magnetic fluidized bed system as tool for the isolation and pre-concentration of EVs in plasma/serum patient samples, several steps/issues have been evaluated including the immobilization chemistry of antibodies on the surface of magnetic particles for increased EV recovery, the dimensions of the chip, the flow rate and sample volume. Capture and release efficiency were evaluated by direct on-chip monitoring of the fluorescence when working with fluorescent samples or by ELISA test. Capturing and release steps were also monitored on anti-tetraspanins antibody microarrays by fluorescence and interferometric detection.

Summary/Conclusion: In summary, we have demonstrated that intact EVs can be released from the first module of the sensing platform and transferred to the second module devoted to nanoparticle sizing and phenotyping

Funding: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no. 766466. INDEX.

PS04.05

Comparison of extracellular vesicles detection by microfluidic plasmonics of gold nano-island and nanocomposite platforms

Muthukumaran Packirisamy[a], Srinivas Bathini[a], Simona Badilescu[b], Duraichelvan Raju[a], Anirban Ghosh[c] and Rodney J Ouellette[c]

[a]Concordia University, Montreal, Canada; [b]Concordia University Montreal, Montreal, Canada; [c]Atlantic Cancer Research Institute (ACRI), Moncton, Canada

Introduction: Extracellular vesicles (EVs) are groups of nanoscale extracellular communication organelles in the order of 30–100 nm, which can be used as disease biomarkers for cancer. In this work, we have developed different platforms for the detection and characterization of EVs by using a localized surface plasmon resonance (LSPR) method based on the sensitivity of the gold plasmon band to the environment of gold nanoparticles.

Methods: EVs from breast cancer cell line (MCF7) are detected and characterized by using a gold nanoparticle-based plasmonic platforms. Here, two different platforms have been developed, a gold nano-island platform on glass substrate and a gold poly(dimethyl) siloxane (Au-PDMS) nanocomposite. A plasmonic sensing protocol is established and carried out by using the two platforms and, subsequently, the procedure is transferred in a microfluidic environment. Gold nanoparticles are first deposited on glass substrates and annealed to form gold nano-islands, whereas the gold nanoparticles were *in-situ* synthesized in a PDMS matrix by the immersion method. EVs are affinity captured by a peptide (Vn96) in this protocol. The two platforms were individually bonded to a PDMS sample having a channel to form a microfluidic device. The entities involved in the biosensing protocol are flown through the channel, and the absorption spectrum is measured after each step.

Results: A graph showing the LSPR shift of the gold plasmon band for different concentrations of EVs is plotted for the two platforms. The exosomes from breast cancer cell line (MCF7)-conditioned media have been grown in a small bioreactor. Comparable results in terms of sensitivity have been found for the two platforms.

Summary/Conclusion: Compared to the macro detection method, the microfluidic detection of EVs proved to be highly reproducible and more sensitive as very small amount of chemicals and EVs are necessary for the analysis.

PS04.06

Dielectrophoretic nanovesicle sorter

Yong-Sang Ryu[a], Avijit Barik[b], Nathan J. Wittenberg[b], Daniel A. Mohr[b] and Sang-Hyun Oh[3]

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Introduction: Extracellular vesicles are membrane-bound particles that play important roles in cellular communications, packaging of genetic material and waste management. An important category of extracellular vesicles, exosomes, are only 30-100 nm in size. To investigate the biological functions of these extracellular vesicles and to use them for applications in diagnostics and drug delivery, rapid isolation with high collection efficiency and selectivity is of great importance. Small unilamellar vesicles (SUVs), as a model type of exosomes, have been extensively exploited to characterize the role of extracellular vesicles during the processes.

Methods: 2.1. Fabrication of 10 nm-width-gap electrode device

2.2. SUV preparation and size characterization

2.3. Dielectrophoresis on nanogap electrodes

Results: Here we demonstrated that dielectrophoresis (DEP) can be used to collect and sort sub-100 nm SUVs, a model of exosomes, based on their size and the electrical properties of their cargo. The DEP platform is based on a 0.8 mm-long, 10 nm-wide gap between gold electrodes, capable of generating ultra-high electric field gradients with low voltages. We determine the DEP trapping threshold voltages as a function of vesicle size for the selective capture. Furthermore, SUVs with different internal conductivities can be sorted by varying DEP frequency.

3. 1. Dielectrophoretic trapping of SUV and size-dependent sorting

3.2. SUV sorting based on internal conductivity.

Summary/Conclusion: Such differential DEP responses may allow the isolation of membrane-free macromolecular aggregates in the presence of empty vesicles down to size ranges of $d \leq 100$ nm without labelling processes required for detection methods used with other separation techniques. Our electronic DEP sorter can readily be applied to diverse biological materials including viruses, proteoliposomes, functionalized nanobeads, DNA molecules and other biomolecules.

Funding: This research was supported by grants from the Minnesota Partnership for Biotechnology and Medical Genomics, MnDrive Research Initiative, NSF through the National Nanotechnology Coordinated Infrastructure (NNCI) program, and internal project of KIST.

PS04.07

A novel capture-and-release platform to isolate extracellular vesicle subpopulations reveals functional heterogeneity among EVs with different surface markers

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Introduction: Extracellular vesicles (EVs) are heterogeneous in terms of size and molecular composition, which may also reflect functional differences. For example, given that the EV surface dictates interactions with their environment, EVs with different surface profiles may be taken up and processed by target cells in different ways. Unfortunately, tools to isolate and functionally compare EV subpopulations based on their surface marker expression are currently not available. Here, we describe a novel capture-and-release platform to separate intact EVs based on specific surface signatures and compare their properties.

Methods: EVs were isolated from MDA-MB-231 cells using size exclusion chromatography. EV subpopulations expressing specific surface markers were captured on magnetic beads and released using a novel release protocol. Released EVs were characterized by western blotting, nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). Uptake of fluorescently labelled EV subpopulations by various cell types was examined using flow cytometry.

Results: Isolated MDA-MB-231 EVs showed typical EV properties, including the presence of EV marker proteins, heterogeneous size distribution (mode size of 120 nm) by NTA and intact, "cup-shaped" morphology as visualized by TEM. When these EVs were subjected to the capture-and-release platform, EV subpopulations with different properties were obtained. Released subpopulations appeared intact as demonstrated by TEM, but differed in their size distribution. Furthermore, EV subpopulations showed different enrichment/depletion patterns of canonical EV proteins as shown by western blot. Lastly, uptake of EVs by target cells differed between EV subpopulations and between target cell types.

Summary/Conclusion: In this work we showcase a novel capture-and-release platform to separate intact EV subpopulations based on their expression of specific surface markers. Using a small panel of antibodies against EV surface markers, we show differences between EV subpopulations in terms of protein composition, size distribution and cellular uptake by target cells. We anticipate that this tool can help to clarify relationships between the surface signature of EVs and their functionality, and facilitate the enrichment of EVs with desirable characteristics for therapeutic purposes.

PS04.08

Nanopillar and nanochannel fabrication via mixed lithography

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Introduction: Extracellular vesicle (EV) sorting and separating by nanostructure is essential to achieve a size-dependent analysis of protein and miRNA inside the vesicles. In this regard, implementation of lab-on-a-chip devices having the EV sorting functionality has been pursued by utilizing the physical properties of the particles.

Methods: Nanopillar array is a useful template for sorting and separating EVs. We report a method of fabricating nanopillar array coupled with large-scale fluidic structures. To do this, we introduce mixed lithography by which both nanometer-scale functional features and large-scale guiding structures are generated in the same level upon 200 mm silicon wafers.
Results: Upon 200 mm silicon wafer, nanometer features are firstly produced by electron beam lithography (EBL) in the extremely localized area which is subsequently connected by the micrometer structures produced by photolithography. By introducing hard-masking oxide layer, we can create the coupled geometry in the same level structure. For the nanometer fluidic channels, we examine wetting of a liquid solution containing fluorescent polystyrene particles.
Summary/Conclusion: We demonstrate EV sorting devices by implementing nanostructures in lab-on-a-chip structure. Our method may offer a way to produce biochips that have versatile functions including sorting and separating EVs.
Funding: This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science & ICT (2017M3A9G8083382).

PS04.09

Towards on-chip EVs separation: a lab-on-chip approach

Lyne Pillemont, Daniel Guneysu, Celine Elie-Caille[a], Wilfrid Boireau[b] and Anne-Marie Gue[c]

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Introduction: Owing to their complexity in size, origin, membrane markers, there is currently no ideal technology available to relate cell-derived microvesicles (EVs) structure and functions. All currently available methods (flow-cytometry, DLS, TRPS, etc.) have limits in their ability to capture the whole diversity of EVs populations and are not amenable to automation and large-scale analysis of numerous samples. In that context, the overall objective of this study is to develop a miniaturized platform allowing the isolation, fractionation and qualification of microvesicles in μL volume.
Methods: Based on previous works (1), we propose a lab-on-chip coupling a hydrodynamic separation module enabling EVs separation according to their size to an affinity-trapping chamber compatible with subsequent SPR and AFM characterization. We designed and fabricated 2.5 × 2.5cm chips enabling the separation of vesicles at tunable cut-off (150-900nm). The proof-of-concept was done using fluorescent calibration particles (polystyrene and melanin resin nanoparticles) biofunctionalized with proteins and mimicking EVs in buffer solution.
Results: Sample was introduced into the chip using a syringe pump or a pressure generator and the filtered sample was simply collected at the chip outlet and redirected towards a biodetection chamber designed as an array of gold plots functionalized with antibodies. We demonstrated the high quality separation of 490 nm nanoparticles from 920 nm particles in concentrated solution (2.109 to 2.1011 particles/μL). Following sorting step, biosynthetic particles were immunocaptured in a miniaturized module of the NBA platform (2, 3) for their subsequent analysis.
Summary/Conclusion: We did the proof-of-concept of on-chip nanoparticles separation and capture demonstrating the ability of miniaturized systems to perform sample fractionation. The tunable properties of the device open the way to a versatile tool for pre-analytical steps of EVs, including sorting and concentration, even in complex media.
Funding: ANR: Agence Nationale de la Recherche

PS04.10

Acoustophoretic-based microfluidic platform for sorting extracellular vesicles

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Introduction: Conventional methods used for isolation of extracellular vesicles (EVs) are time-consuming, produce low purity samples and may change the structure of EVs. To address these problems, microfluidics-based EV isolation methods have been introduced. In particular, acoustic-based cell isolation (functioning based on size, density and compressibility differences of bioparticles and medium) have shown potentials. However, the geometrical and operational parameters of such a platform still need to be optimized to produce high throughput and reproducible results. This study focuses on the optimization of an acoustophoretic-based microfluidic platform using first colloidal particles following by EVs isolated from culture media from cancer cell lines. The results are compared against the

conventional method to show high yield and purity of the proposed platform.

Methods: The acoustic pressure field can be generated inside a microchannel by applying a voltage to patterned interdigital transducers fingers on the surface of piezoelectric materials. Due to such a field, bioparticles are deflected (and hence sorted) at different points along the microchannel depending on their volumes. Soft lithography and etching processes are used for fabrication of microchannel and transducers of the platform.

Results: To optimize the geometry and operational parameters of the platform, polystyrene (PS) particles are first used as they have similar size, density and compressibility of the components in the body fluid samples. The results showed that 90% of PS particles are deflected at a frequency of 26.5 MHz and the input voltage of 10 Vpp. Using these parameters, we are then able to sort EVs from cell culture media into size ranges between 500–1000 nm. The size of each sorted vial is characterized by nanoparticle tracking analysis and shown a size separation resolution of 500 nm and a throughput of 4 uL/min.

Summary/Conclusion: Acoustofluidics-based separation results show the size separation resolution of 500 nm and a throughput of 4 uL/min, indicating the protentional of such a technique as a non-invasive, label-free and effective EV purification method.

Funding: This work was supported by the University of British Columbia Eminence fund.

PS04.11

Proteomic and miRNA analysis of highly purified extracellular vesicles recovery using immunoaffinity purification and ultracentrifugation from serum, plasma and urine

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Miraca Research Institute G.K., Hachioji-shi, Japan

Introduction: Exosomes, one of extracellular vesicles, are secreted into extracellular fluids from all types of cells via endosomal pathway and found in most body fluids including blood and urine. Exosomes are reportedly associated with various disease conditions including cancer metastasis and vascularization. Although exosomes seem to be promising biomarkers, methods to isolate and quantify exosomes still remain controversial. Conventionally used methods include ultracentrifugation (UC), polymer precipitation and immunoaffinity purification (IP) using surface marker antibodies. In addition, obtained exosomes from certain types of specimens, urine in particular, is extremely difficult.

In this study, we aimed to establish a method to efficiently recover exosomes from serum, plasma and urine using IP and UC method, considering practical use at the clinical site.

Methods: Antibodies against tetraspanins and IP condition were established and used to isolate exosomes from serum, plasma and urine. Obtained exosomes were subjected to immunoblotting, nanoparticle tracking analysis (NTA), proteomic analysis, internalization assay and 3D-Gene miRNA microarray.

Results: Immunoblotting and NTA revealed the recovery of highly pure exosomes from serum and plasma with increased efficiency by our IP method. Our method was successful in recovering exosomes from urine specimens, whereas commercialized antibodies failed to do so. Internalization assay showed that uptake rate of exosomes isolated from conditioned medium using our method were similar to that of exosomes isolated using conventional method. Number of identified proteins has increased, whereas the detection of nonspecific proteins decreased by our method. Expression profiles of miRNAs from our obtained exosomes differed from that obtained by conventional isolation method.

Summary/Conclusion: Our established exosome purification methods are capable of efficiently recovering exosomes from serum and plasma in addition to urine specimens. Our approach can be readily automated to isolate exosomes from specimens, which could contribute to therapeutic application of exosomes and biomarker detection.

PS04.12

Capture and release of extracellular vesicles in tens of μL samples for ocular neuroprotection studies

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Introduction: The incidence of eye diseases is on the rise with increasing longevity and use of 3C products. However, treatments for several eye diseases, such as vision-threatening glaucoma and age-related macular lesions, offer only symptomatic control with no curative options. Extracellular vesicles (EVs) are cell-derived vesicles that have been shown to play a role in intercellular communication, immune regulation, extracellular matrix turnover, stem cell division/differentiation, neovascularization and cellular waste removal. At present, ocular EV studies remain rare

mainly due to the challenges associated with accessing and processing minute ocular samples.

Methods: In this work, we collected EVs from Sprague Dawley rat intraocular samples after non-arteritic anterior ischaemic optic neuropathy (NAION) induction. 30 μL ocular fluid collected at day 0, 0.25, 1, 3 and 7 after NAION induction was applied to each paper-based device. Long-wavelength UV light (360 nm) was utilized to break the photolabile crosslinker and release captured EVs for subsequent analyses.

Results: RNA molecules contained in captured CD63 + EVs were extracted, and the next generation sequencing (NGS) results showed that more anti-inflammatory M2 miRNAs were present in NAION samples than in sham controls. In addition, we have identified 53 miRNAs that showed more than two-fold changes in expression during the natural course of recovery after NAION. These miRNAs included pro-inflammatory M1-related miRNAs (miR-184, miR-3473, let-7c-5p, miR-124, miR-125a-5p, miR-210-3p) and anti-inflammatory M2-related miRNAs (miR-31a-5p, miR-99a-5p, let-7i-5p, miR-204-5p, miR-16-5p). Interestingly, M1-related miRNAs exhibited a biphasic expression that peaked at day 1 and then elevated again at day 7, whereas M2-related miRNAs were upregulated at day 7 from NAION to achieve putative neuroprotection effects.

Summary/Conclusion: We have developed an easy and fast method capable of collecting and releasing EVs from low-volume samples. The quantity and quality of miRNA extracted is enough for NGS analysis.

Funding: Taiwan Ministry of Science & Technology (MOST 106–2628-E-007–010-MY3) and the Taiwan Ministry of Education (Higher Education Sprout Project: Grant No. 107Q2713E1).

PS04.13=OWP3.04

An integrated microfluidic device for selective exosome isolation from human plasma

Hogyeong Gwak[a], Junmoo Kim[b], Leila Kashefi-Kheyrabadi[b], Seung-Il Kim[b], Kyung-A Hyun[b] and Hyo-Il Jung[b]

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Introduction: Extracellular vesicles released by many cell types circulate in blood vessel and play a key role in intercellular communication. Exosomes are 30–150 nm membrane vesicles and are also shed by both normal and cancer cells. Cancer cells are known as very heterogeneous, so exosomes are also heterogeneous and have different surface expression markers. Cancer-derived exosomes contain unique cargo determined by the molecular characteristics of cancer cells. Therefore, it is very important to selectively separate exosomes depending on surface expression for downstream analysis. We designed an integrated microfluidic chip for selective exosome isolation. The microfluidic chip consists of Hoof Structure (HS) for mixing exosomes and two different sized aptamer-coated particles and Multi-Orifice Flow Fractionation (MOFF) for separating each particle.

Methods: Biotinylated EpCAM aptamer was immobilized on the surface of 7 μm streptavidin-coated polystyrene particle and HER2 on 15 μm. The HS has the circular expansion channel on the 1st layer to generate expansion vortices and the two curvature channels on the 2nd layer to make chaotic advection. It makes transverse flow and mixes two particles without particle focusing phenomenon. The 100-nm (exosome), 7-μm and 15-μm fluorescence particles were used to test mixing performance between exosomes and particles in the HS. The MOFF was designed by a series of contraction/expansion microchannels for continuous size-based separation. Separation performance was tested by using the 7-μm and 15-μm fluorescence microparticles in the MOFF.

Results: The mixing efficiency was the highest at the flow rate 150 μl/min. Each exosome was continuously captured by aptamer-conjugated particle in the HS channel. The capture efficiency of EpCAM positive exosome was 96.9% and HER 2 was 68.09%. Two particles were separated in the integrated microfluidic device at the same flow rate. 96.26% of 15 μm microparticles were positioned into the centre of the channel, and 89.48% of 7 μm microparticles were separated on both sides of the channel.

Summary/conclusion: Each exosome was continuously captured by mixing aptamer-conjugated particle in the HS. Exosome-conjugated microparticles were successfully separated by inertial force in MOFF. This analysis of each exosome will shed light on diagnosis and therapy of cancers.

PS05: EV Protein Biomarkers
Chairs: Seiko Ikezu; Yusuke Yoshioka
Location: Level 3, Hall A

15:00–16:00

PS05.03

Caveolin-1 reduces in extracellular vesicles derived from lung cancer tissue and plasma and associates with cancer cell migration

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Introduction: Early diagnosis is of significance meaning for lung cancer. Extracellular vesicles (EVs) are a new kind of diagnostic biomarkers with great potential. However, the discovery of biomarkers based on EVs remains disturbed by EVs from cells disassociated with lung cancer. If biomarkers, we suggest, can be screened based on EVs from cancer tissue and validated in plasma, discovered biomarkers may combine good specificity and practicability in clinical practice.
Methods: Thirteen Lung cancer tissues and 71 plasma samples (47 early stage lung cancer patients, 9 advanced stage lung cancer patients and 15 healthy controls) were collected from Nang Fang Hospital. Our research was approved and supervised by the Medical Ethics Committee of Nan Fang Hospital. EVs were purified from lung cancer tissues and paracancerous tissues and characterized by LC MS/MS; protein profiles of two groups were compared and Caveolin-1 was picked out in differentially expressed proteins. With high-sensitivity flow cytometry, the diagnostic performance of Caveolin-1 was validated in 79 plasma samples. In cell line experiments, Caveolin-1 on EVs was blocked by antibody, and the migration of EVs stimulating cancer cells was evaluated by transwell.
Results: We determined profiles of EVs in lung cancer tissue and paracancerous tissue separately. Combined bioinformatics analysis and western blotting verification, Caveolin-1 was chosen as candidate biomarker and verified by western blotting in six plasma samples. Subsequently, Caveolin-1 was evaluated in 79 plasma samples. Caveolin-1 was significantly decreased in lung cancer patients and the area under curve of ROC reached 0.958 in diagnosis of cancer patients and healthy controls. Furthermore, we observed the biological function of Caveolin-1 on EVs with cell line. When cancer cells were co-cultured with EVs, the movement of cancer cells stimulated by antibody-blocked EVs was increased.
Summary/Conclusion: Our study indicated Caveolin-1 on EVs can inhibit cancer cell movement. Cancer cells promote the movement of other cells by reducing Caveolin-1 on EVs. Caveolin-1 significantly decreased in cancer tissues and patients' plasma. Caveolin-1 may serve as a potential marker for the diagnosis in lung cancer diagnosis.
Funding: This study was supported by grants from the National Natural Science Foundation of China (81371901).

PS05.04

DNA assembly assisting magnetic fluorescence nanosensor based on aggregation-induced emission probe/graphene oxide for cancerous exosome analysis

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Introduction: Exosomes are emerging as non-invasive diagnostic biomarkers of cancer because they carry biomolecules that include proteins and nucleic acids for intercellular communication. Assessing special exosome surface proteins provides a powerful means of identifying the origins of their cancerous parental cells. Glypican-1 (GPC-1), an exosomal membrane protein, was discovered to have much higher expression on the cancerous exosomes than the noncancerous for the early diagnosis of pancreatic, breast and colorectal cancer. However, quantification of low concentrations of specific exosomes present in very small volumes of clinical samples remains a challenge.
Methods: Herein, we proposed a magnetic fluorescence nanosensor based on GPC-1 antibody-functionalized magnetic microcarriers for GPC-1(+) exosome subpopulation isolation and detection. And the recognition signal was transformed into the formation of free-state DNA nanostructure by the trigger containing CD63 aptamer. To increase the sensitivity of this method, a "Y" shape DNA self-assembly nanostructure amplification strategy was adapted to assist aggregation-induced emission probe/graphene oxide (AIE/GO) "turn-on"

fluorescence reporting system to realize the label-free and ultracentrifugation-free quantitative analysis of GPC-1(+) exosome subpopulation of breast cancer in a homogeneous.

Results: We optimized the reaction conditions and evaluated the detection performance of the method, such as specificity, sensitivity and linear range. Under optimal conditions, the results show that this magnetic fluorescence nanosensor could distinguish breast cancer exosomes from five other types of cancerous exosomes, and the linear range of detection for breast cancer exosomes is estimated to be 7.8 × 104–3.9 × 109 exosomes/μL with a detection of limit (LOD) of 6.56 × 104 exosomes/μL. We demonstrated the application of the magnetic fluorescence nanosensor in quantitative detection of exosomes in plasma samples directly from breast cancer patients.

Summary/Conclusion: This magnetic fluorescence nanosensor is expected to become a powerful tool for rapid and simple cancer liquid biopsy.

Funding: This study was financed by grants from the National Natural Science Foundation of China (81371901, 81702100) and the Science and Technology Planning Project of Guangdong Province (2017A020215123)

PS05.05

Quantitative multiparameter analysis of individual urinary extracellular vesicles via a laboratory-built nano-flow cytometer

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Introduction: Urinary extracellular vesicles (uEVs) have attracted much attention as a source of non-invasive biomarkers. To exploit their prominent potential in the diagnosis of urinary tract diseases including urinary cancers, an in-depth study of uEVs at the single-particle level is important. Employing a laboratory-built nano-flow cytometer (nFCM) that facilitates multiparameter analysis of single EVs as small as 40 nm, here we report quantitative measurement of size distribution, particle concentration, purity, lipid membrane, nucleic acids and surface proteins of uEVs.

Methods: uEVs were isolated from mid-stream urine samples collected from healthy donors via differential ultracentrifugation (UC). Monodisperse silica nanoparticles were used as the size reference standards for the size distribution measurement of uEVs via light scattering detection. By using fluorescent silica nanoparticles of known particle concentration as the internal standard, particle concentration of uEVs was measured via single particle enumeration. The purity of uEVs in the isolates was examined by measuring the particle concentration before and after Triton X-100 treatment. Lipid-membrane was labelled with PKH26 and PKH67. Subpopulation of uEVs expressing specific surface proteins were analysed via immunofluorescent staining. SYTO 16, a cell-permeant stain, was used to stain the nucleic acids of uEVs before and after DNase I treatment.

Results: The concentration of uEVs was determined around 10^9 particles/mL in urine, and the purity of isolated uEVs via UC was above 90%. Comparing with the light scattering signal of single EVs, the lipid dye labelling efficiency was found to be around 90%. Considering the purity of EVs, we conclude that almost all the uEVs can be stained by lipid dyes. We also found that ~30% of uEVs expressing CD9, CD63, CD81 or TSG101 on their surface. The ratio of uEVs lightened up by SYTO 16 decreased from 16% to 10% after DNase I treatment, which indicates that part of the DNA resides on the outer membrane surface of uEVs.

Summary/Conclusion: The laboratory-built nFCM is applicable to the multiparameter biochemical analysis of individual uEVs via protein, lipid and nucleic acid staining. We expect nFCM will facilitate more in-depth studies of uEVs and aid the development of clinical diagnosis with uEVs.

PS05.06

Proteomic profiling of urinary exosomes for potential predictors of albuminuria in subjects with diabetes

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Introduction: Albuminuria is considered to be an important clinical hallmark for renal diseases. However, it has limited ability to predict the earliest stages of diabetic nephropathy. Early biomarker potential of urinary exosomes (UE) for renal disease has been highlighted by us and others. We carried out proteomic profiling of UE followed by a longitudinal follow-up study to determine potential predictors of albuminuria in subjects with type-1 diabetes (T1D).

Methods: In study-1, proteomic profiling of UE from T1D with or without albuminuria (urine albumin to creatinine ratio between 30–300 mg/g, n = 3/group) was performed using two-dimensional differential gel electrophoresis (2D-DIGE). Diagnostic potential of one

of the identified UE protein to predict incidence of microalbuminuria was determined. For this, 29 T1D subjects without albuminuria were followed for ~4.5 years (study-2). Urine microalbumin, serum creatinine and HbA1C were analysed.

Results: 2D-DIGE revealed a total number of 592 differential protein spots between T1D subjects with or without albuminuria. The MASCOT search for 26 selected spots revealed 14 proteins associated with nephropathy, including Wilms' tumour 1 (WT1) protein. At the end of 4.5 years of follow-up, 9 subjects (out of total 19) progressed to albuminuria in WT1-positive group (presence of WT-1 in UE at the time of recruitment) as opposed to 1 in WT1 negative group. Both groups had statistically similar diabetes duration, age, % HbA1c and estimated GFR at the baseline.

Summary/Conclusion: Urinary exosomal protein could help in categorizing diabetic subjects that may go on to develop nephropathy.

Funding: Funded by intramural, DST, Govt. of India.

PS05.07

Identification of exosomal biomarkers in urine for human prostate cancer

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Introduction: Prostate cancer (CaP) is the second leading cause of cancer-related death in males. Identification of novel biomarkers is important for the early detection of CaP. Exosomes are small membrane-bound vesicles released from most cell types including cancer cells. Exosomes play a key role in intercellular signalling and potentially play a role in tumorigenesis and cancer progression. In this study, we investigated differential urinary exosomal proteins in CaP patients compared to healthy controls by mass spectrometry.

Methods: Midstream spot urine samples from CaP (n = 20) and benign prostatic hyperplasia (BPH; n = 10) patients were obtained, as well as urine from age- and sex-matched controls (n = 10). Urinary exosomes were isolated using the Total Exosome Isolation Reagent (invitrogen). The presence of exosomes was evaluated by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Exosomal markers including TSG101, CD9, CD63 and CD81 were validated by western blotting (WB) and flow cytometry (FC). High-throughput LC-MS/MS-based label-free quantification was performed on Q Exactive to identify proteins in the exosomes. Three biomarker candidates VCAM1, IL18BP and S100A6 were selected for further validation in urine exosome samples from a separate cohort of CaP patients and CaP cell lines by WB.

Results: We successfully isolated exosomes from human urine, which were further validated by TEM, NTA, WB and FC. In total, 1330 proteins were identified through LC-MS/MS. Among them, 596 proteins were differentially expressed between CaP and normal controls. According to statistical analysis, a focus list of 37 proteins, including 17 upregulated and 20 downregulated proteins was revealed as dysregulated candidates in urinary exosomes for CaP. The validation of potential biomarkers including VCAM1, IL18BP and S100A6 showed that the levels of these proteins were higher in CaP cell lines including PC-3, PC-3M, DU145 and LNCaP compared to the normal prostate cell line RWPE-1. In addition, the expression level of IL18BP was higher in urinary exosomes from CaP patients compared to healthy controls.

Summary/Conclusion: Urinary exosomes harbour informative proteins that might be used for the early detection of CaP or monitoring its progression through a non-invasive way.

Funding: ARC-Linkage Grant

PS05.09

Optimization of exosome isolation and ELISA method for identification of novel cancer biomarkers

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Introduction: Exosomes are a type of extracellular vesicles with diameter of 30–150 nm secreted by cell and circulate in blood abundantly. Especially, cancer-cell-derived exosomes contain oncogenic molecules that can be novel biomarker for cancer diagnosis. Recent compelling issue of cancer patients is the immune system that is negatively regulated by cancer-cell-derived exosomes. Therefore, first we have to optimize exosome isolation methods and ELISA methods to analyse exosome's constituents precisely. Through this method, we can screen several candidates which contain in cancer-cell-derived exosomes to identify novel biomarkers for cancer prediction.

Methods: Exosomes were isolated from cancer patients' plasma using serial centrifugation method. For western blot analysis, we loaded exosomes to observe existence and difference in the expression of protein between

cancer patients' and healthy controls'. And using exosomes each well in 96-well plate, sandwich ELISA was performed to measure protein level of exosomes from cancer patients' and healthy controls'. We also made mouse xenograft models to find the correlation between exosomal protein level and tumour burden.

Results: We optimized isolation method to purify exosomes and to minimize sample variation, and we optimized ELISA method using well-known exosomal surface biomarkers and confirmed assay stability. By optimization of exosome isolation and ELISA method, we built finding system for novel cancer biomarker which is expected significantly overexpressed in exosomes from cancer patients' plasma compared to healthy controls'. In addition, we checked the level of exosomal surface protein's correlation with tumour burden, therefore prove possibility as novel cancer biomarkers.

Summary/Conclusion: Based on our results, we optimized our own finding system and identified novel cancer biomarkers.

Funding: This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science & ICT (2017M3A9G8083382) and by the National Research Foundation of Korea (NRF) grant funded by the Korea government (2014R1A5A2009242).

PS05.10

Thyrotropin receptor-positive exosomes alleviate autoantibody-mediated stimulation of cAMP production

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Introduction: Exosomes or extracellular vesicles secreted from cells play a variety of roles in both physiological and pathological processes. In Graves' disease (GD), autoantibodies bind to thyrotropin receptor (TSHR) on thyroid follicular epithelial cells, stimulating thyroid growth and thyroid hormone synthesis and secretion through cAMP production. In this study, we examined if exosomes expressing TSHR are secreted from thyroid cells and defined their roles in GD.

Methods: Exosomes by differential centrifugation from the culture medium of NTHY-ori 3-1 human thyroid follicular epithelial cell line and 8305C, 8505C and FTC133 thyroid carcinoma cell lines. Western blot analysis was performed to detect TSHR in cell lysates and exosomes. Human embryonic kidney HEK293 cells (HEK) overexpressing TSHR (HEK/TSHR) were established for the functional analysis of TSHR exosomes. Using exosomes isolated from HEK and HEK/TSHR cells, *in vitro* binding capacity of a human monoclonal autoantibody (M22) to TSHR exosomes and their effect on M22-mediated stimulation of intracellular cAMP production in HEK/TSHR cells were studied. Human recombinant TSHR chimera capable of binding to M22 was used as a positive control.

Results: TSHR was detected in exosomes from cancer cells as well as normal epithelial cells. The binding assay demonstrated that M22 dose-dependently bound to TSHR exosomes. M22 stimulated intracellular cAMP production in HEK/TSHR cells in a dose-dependent manner. Exosomes from HEK/TSHR cells but not those from HEK cells significantly reduced cAMP production activated by M22 in HEK/TSHR cells. A similar inhibitory effect was observed for human recombinant TSHR chimera.

Summary/Conclusion: Our results suggest that TSHR exosomes may be secreted from normal and cancerous thyroid epithelial cells. In the thyroid gland of patients with GD, TSHR exosomes may exert a decoy effect by sequestering M22, alleviating autoantibody-stimulated cAMP production.

Funding: There is nothing to disclose.

PS05.11=OWP3.06

***In vitro* and *in vivo* investigation of extracellular vesicles (EVs) as biomarker carriers in the diagnosis of early Alzheimer's disease**

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Introduction: Extracellular vesicles (EVs) represent an ideal source of biomarkers due to their role in cellular communication and their ability to carry protein aggregates. The most investigated EVs are exosomes, active entities secreted from cells and able to cross the blood–brain barrier. Several neurodegeneration-involved molecules may undergo intercellular spreading through exosome release. In Alzheimer's disease (AD), before clinical signs appear, several proteins implicated in exo- and endocytic pathways are altered. In this scenario, the identification of a correlation between

variations in proteins carried by EVs and the progression of AD is the main aim of our project.

Methods: We performed exosome isolation and characterization from H4-SW glioma cells (a cell model featuring mutated β-amyloid overexpression), as well as in mouse- (triple-transgenic mouse model for familial AD) and human-plasma samples (Mild Cognitive Impairment (MCI) and AD subjects). In every case a differential centrifugation protocol was applied and exosomes were then characterized using Nanoparticle Tracking Analysis with the NanoSight. We then explored exosome content, specifically Amyloid Precursor Protein (APP) and its proteolytic fragments, Microtubule Associated Protein Tau (tau), Progranulin (PGRN protein), Soluble Triggering Receptor Expressed on Myeloid Cells 2 (sTREM2) and α-synuclein (α-syn), using Western blot and ELISA. L1CAM and CD63 were evaluated to define the neural-derived exosomes amount in human samples. All the samples were collected after ethical committee approval respecting Helsinki's declaration. Informed consents were provided by all the subjects.

Results: Our preliminary results show that APP, PGRN, sTREM2 are carried by H4- and human plasma-derived EVs. H4-SW cell-culture medium and 3Tg mouse plasma had a decrease in the EVs number release (≈1*10e8 EVs/ml) in comparison to control (≈7*10e8 EVs/ml). This decrease was not found in human plasma samples.

Summary/conclusion: EVs purified from H4-glioma cellular AD model, 3xTg mouse-, MCI- and AD-plasma samples carry proteins relevant for neurodegenerative diseases (NDs). EVs release is reduced in cellular and animal AD-models.

Funding: Horizon 2020 Marie Sklodowska-Curie Innovative Training Networks – Blood Biomarker-based Diagnostic Tools for Early Stage Alzheimer's Disease.

PS06: Advancing EV Studies in Biological Samples
Chairs: Peter Kurre; J. Bryan Byrd
Location: Level 3, Hall A

15:00–16:00

PS06.01

AR-V7 in urinary EVs of patients with prostate cancer

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Introduction: Prostate cancer is the most common cancer affecting men and a leading cause of cancer deaths. Almost all patients initially respond to androgen deprivation therapy but inevitably progress to a lethal stage of disease, termed castration-resistant prostate cancer (CRPC). Androgen-receptor splice variant (AR-V7) is associated with CRPC and resistance to anti-androgen therapy. Despite its clinical importance, the lack of efficient methods for AR-V7 analysis remains a challenge for broader use of this marker in routine clinical practice. Here we suggest a practical and non-invasive liquid biopsy method for analysis of AR-V7 in the RNA of urine-derived extracellular vesicles (EVs) without the need for blood withdrawal.
Methods: Urine samples were collected from patients at Pusan National University Hospital (PNUH). The study protocol was reviewed and approved by the Institutional Review Board of PNUH and UNIST, and written informed consent was obtained from all subjects. All patients that progressed to CRPC underwent docetaxel-based chemotherapy. Using a newly upgraded centrifugal microfluidic device for size-based EV isolation, rapid enrichment of EVs (< 30 min) from each 4 mL of urine was accomplished. Followed by mRNA extraction, and AR-V7 and androgen-receptor full-length (AR-FL) mRNA levels were quantified by droplet digital polymerase chain reaction (ddPCR). Furthermore, protein and mRNA expression of EVs isolated from blood plasma are compared together.
Results: Higher AR-V7 and lower AR-FL expression were detected in urine-derived EVs from 14 patients with CRPC (0–217.0 and 12.9–562.5 copies/mL, respectively) than in those from 22 patients with hormone-sensitive prostate cancer (HSPC, 0–24.1 and 6.2–9053.6 copies/mL, respectively). Also, we found that AR-V7 transcript levels and the AR-V7/AR-FL ratio in urinary EVs were higher in patients with advanced prostate cancer.
Summary/Conclusion: This study demonstrates that mRNA of urine-derived EVs is a reliable source for AR-V7 expression analysis, suggesting a simple and promising approach to liquid biopsy with a great potential for therapeutic impact on prostate cancer.
Funding: This study is funded by Ministry of Health and Welfare (HI12C1845) and Institute for Basic Science (IBS-R020-D1), Republic of Korea.

PS06.02

Microvesicles are absorbed on the surface of extracorporeal membrane oxygenation circuit tubing

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Introduction: There is an increasing interest in potential role of microvesicles (MVs) as non-invasive biomarkers for acute critical care diseases. Clinically, extracorporeal membrane oxygenation (ECMO) draws attention in critical care, and MVs are considered to be involved in the development of adverse events in critically ill patients. However, the dynamics of MVs within ECMO circuit, in particular MV absorption on the biomaterial, has not been systematically studied. The purpose of this study was to explore whether MVs are absorbed by ECMO circuit tubing.
Methods: Granulocytes were isolated from healthy volunteer blood using density-gradient centrifugation. MV production was induced by treatment with calcium ionophore for 20 min, and MVs were quantified on flow cytometry (FACS) based on their size and surface expression of CD11b and CD66b. MVs were then purified by differential centrifugation and dissolved with DMEM. Thereafter, 0.5 to 1.0 × 107 MVs were administered to either ECMO circuit tubing (material group) or Eppendorf tubes (control group) with their lid removed and re-sealed with paraffin film.

MV solution was rotated for 6 h, and small volume of sample was regularly taken in 1–2 h interval for MV quantification by FACS.
Results: The number of MVs in the material group was significantly decreased over time, compared to the control group (64% reduction vs. 22% reduction at 6 h. Significant interaction (n = 3, $p < 0.05$) between time and treatment by 2-way repeated measures ANOVA).
Summary/Conclusion: ECMO circuit is known to absorb bioactive drugs and mediators, and our results showed that ECMO circuit also impacts on the dynamics of circulating MVs. Further investigation should explore the effects and mechanisms of MV absorption/production induced by the biomaterial-cell interaction.

PS06.03

Measurement of physical properties of exosome by atomic force microscope

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Introduction: It has been shown that exoxome can be used as a drug delivery system (DDS), and exosome in blood / body fluid has been studied as a new diagnosis method for various diseases. The existence of various proteins and sugar chains such as CD antigen, integrin and ligand has been confirmed in the membrane of exosome. However, physical properties of exosome and their contribution for exosome-mediated phenomenon have not been clarified yet. There are many reports showing the morphology of exosomes by electron microscopy; however, it is necessary for chemical fixation of sample and vacuum environment. Therefore, the form of exosome by electron microscopic observation and the form of exosome in liquid may be different. The AFM is an equipment that can observe exosome in liquid (physiological saline, culture liquid). In recent years, biomolecular property measurement by AFM has been increasing (Kogure et al., BPS, 2018).
Methods: We used SPM-9700HT (Shimadzu Corporation) for AFM measurement. Measurement mode is dynamic mode and force curve mapping measurement. Exosomes were isolated from human breast cancer cell line (MDA-MB-231) and mouse breast cancer cell line (4T1) (Kosaka et al., JBC, 2013) by ultracentrifugation.
Results: Physical properties of exosome were measured using AFM observation and force curve measurement. Exosomes derived from low metastatic strains derived from human breast cancer cell lines showed a relatively smooth surface. Exosomes derived from highly metastatic strains have layers or chain-like substances having a thickness of 10–20 nm. In addition, its physical property was soft. Furthermore, it was found that exosomes derived from nSMase2 knock-down 4T1 cells are harder than exosome from wild type or nSMase2 over-expressing 4T1 cells.
Summary/Conclusion: Now, we are investigating how these properties are related to exosome characteristics in cancer malignancy.

PS06.04

Stability of human salivary extracellular vesicles under gastrointestinal tract conditions

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Introduction: Human saliva plays an important role as front-line of body defence. Extracellular vesicles (EVs) are secreted from various types of cells and it is recognized that they are involved in intercellular communication via delivering their contents. We have isolated EVs with dipeptidyl peptidase IV (DPP IV) from human whole saliva, whereas little is currently known about the fate of secreted salivary EVs. In the present study, we investigated morphological stability of salivary EVs and chemical stability of proteins associated with the EVs in simulated gastrointestinal (GI) fluids.
Methods: Human whole saliva was collected from healthy volunteers. Salivary EVs were separated by size-exclusion chromatography. For simulated gastric fluids or intestinal fluids treatment, indicated concentration of pepsin (pH3.0) or pancreatin (pH7.4) was added to EV samples, respectively. For bile acid treatment, sodium cholate (pH7.4) was added. After the incubation, the treated samples were then subjected to SDS-PAGE, western blot analysis, DPP IV activity measurement, dynamic light scattering study or observation by a transmission electron microscope.
Results: Salivary EVs were morphologically stable under simulated gastric fluids with pepsin and simulated intestinal environment using pancreatin. While some proteins associated with surface of the EVs, such as mucin 5B and CD9, were digested with these treatments, inside components such as Alix and TSG101 were resistant. Even though DPP IV is

oriented outside, it was not digested and retained its enzymatic activity. Thus, membrane integrity was intact and internal components were retained in digestive enzymes. Morphological changes and solubilization of proteins in the EVs scarcely occurred after treatment with physiological concentration of sodium cholate. Membrane integrity was destroyed with increasing concentration of sodium cholate. However, components of the vesicles were not completely solubilized at higher concentration of sodium cholate.
Summary/Conclusion: These results suggest that salivary EVs are stable and functional in GI tract. This study would help to elucidate their potential pathophysiological roles in GI tract.
Funding: This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 16K08348.

PS06.05

The factor affecting to the accuracy of extracellular small non-coding RNA biomarkers

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Introduction: Extracellular small non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), isoforms of microRNAs (isomiRs), tRNA-derived fragments (tRFs) and others, are known as regulator of gene expression for cell metabolism. They are released into body fluid from various cells with extracellular vesicles (EVs) including exosomes. In recent studies, some extracellular miRNAs and tRFs in blood were reported as novel biomarkers for diseases. In this study, we investigated the factor affecting to the accuracy of extracellular small ncRNA biomarkers such as miRNA and tRFs for next generation sequencing (NGS)-based detection.
Methods: Blood was collected from the patients who provided written informed consent to participate in the study (approved by IRB of Hiroshima University). Serum were isolated and stored at −80°C. EVs in the cell culture supernatant were collected after culture in DMEM with FBS followed by one-day additional culture without FBS. Total small RNAs were purified by using miRNeasy Mini Kit (Qiagen). EVs, including exosomes, were isolated by using Total Exosome Isolation Kit (Thermo Fisher Scientific). NGS was performed by using Ion S5 (Thermo Fisher Scientific). We analysed the sequence data of small ncRNAs (15-55 nt) with software, CLC Genomics and JMP.
Results: We found that most of the extracellular small ncRNAs in serum consisted of miRNA, isomiRs and tRFs. Especially, most of ncRNAs in EVs were tRFs. Several isomiRs and tRFs were expressed specifically in serum from cancer patients. Some of them were also observed in EVs from cultured cancer cell lines. EV-free ncRNAs were decreased, and ncRNAs with EVs were increased in blood during long 4°C storage after blood sampling.
Summary/Conclusion: The expression profile of the extracellular small ncRNAs is changed during storage at 4°C after blood sampling. It may affect the accuracy of extracellular small non-coding RNA biomarkers.
Funding: This research is partially supported by the “Development program of microRNA measurement technology foundation in body fluid” from Japan Agency for Medical Research and development, AMED.

PS06.06

Generation of reference material for flow cytometric detection of extracellular vesicles

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Introduction: Extracellular vesicle (EV)-related technologies have been developing rapidly over the past few years and substantial growth is expected for the market as they get integrated into the fields of liquid biopsy, precision and regenerative medicine. NIBSC as a designated WHO standardization laboratory is actively developing methods that in the future may allow the production of diagnostic and therapeutic EV reference material for clinical and pre-clinical use. As flow cytometry enables characterization of EV populations down to single-event level, it has been adapted as a meaningful tool in characterizing EV isolates. High-throughput and multiparameter analysis of EV are crucial to further advance the ability to characterize these particles.
Methods: EVs from plasma samples were isolated using several methods and their morphology and molecular content was assessed. The effects of freeze-drying were investigated to explore a possibility of long-term storage of EV-reference material that has been labelled in that way for flow cytometric analysis.
Results: The populations of submicron EVs could be detected using commercially available flow cytometers only when fluorescence and not light scatter triggered detection was used. The labelling with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester followed

by removal of unbound dye was efficient enough to robustly label single EVs without generating label-associated artefacts. Freeze-drying process had some effects on morphology but not molecular content of EV preperations.

Summary/Conclusion: Efficient labelling and preservation of pure populations of EVs present a viable option for the development of a stable monodispersed reference material that can be used as positive control or calibrant of flow cytometers used for analysing submicron populations.

PS06.07

Comparison of serum and plasma as a source of blood extracellular vesicles reveals possible contamination of serum with platelet-derived particles produced during coagulation

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Introduction: Extracellular vesicles (EVs), including exosomes and microvesicles, are released from cells to extracellular environment, and can be found in several biological fluids, such as blood, cerebrospinal fluid and urine. Among them, blood-derived EVs are expected to offer a more efficient and faster diagnostic method for neurologic diseases than conventional diagnosis. Although serum and plasma are utilized as a source of blood EVs, it still remains unknown whether there are differences in EVs derived from serum and plasma. In this study, we performed a series of experiments to see the differences between serum and plasma EVs.

Methods: Whole blood was obtained from 9-week-old mice. Serum was collected from the supernatant of the clotted blood. Plasma was collected from the blood treated with EDTA. EVs were isolated from serum and plasma using ultracentrifugation method. The morphology of EVs was analysed by electron microscopy, and the particle numbers and the diameter were measured by nanoparticle tracking analysis (NTA). The protein contents of EVs were analysed by LC-MS/MS and western blotting.

Results: NTA measurement revealed that the particle numbers in the EV fraction isolated from serum are ~2-fold larger than those derived from plasma ($p < 0.01$, Student's t-test), while the particle diameter showed no difference between serum and plasma EVs. LC-MS/MS analysis of EVs identified total 520 proteins, of which 317 proteins were detected in both serum- and plasma-derived EVs, while 189 proteins and 14 proteins were detected only in serum- and plasma-derived EVs, respectively. Interestingly, platelet-associated proteins were specifically detected in serum-derived EVs.

Summary/Conclusion: We found that serum has the larger number of EVs than plasma, despite of the same volume of blood. The existence of the platelet-specific proteins detected in serum-derived EVs implies that serum may be contaminated with platelet-derived nanoparticles, which are reported to be produced during coagulation.

PS06.08

Evaluation of stability maintenance of extracellular vesicles upon storage temperature and period

Eun Kyoung Shin[a], Jae Min Cha[b], Mi Jeong Oh[a], Eun Hee Kim[a] and Oh Young Bang[c]

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Introduction: Extracellular vesicles (EVs) secreted from stem cells are bilipid-layered and nano-sized, retaining medicinal potency equivalent to that of stem cells. As much interest in clinical use of therapeutic EVs has been increasingly gained in the fields, however, few studies have been conducted regarding optimal storage and shipping conditions for EVs, which are critical to commercialize EVs as a medicinal product. In this study, we tested the maintenance efficiency of EVs in terms of physical stability and proteomic/genomic contents of EVs in the following storage conditions: (1) 4°C during the 28-day of short-term period and (2) −80°C during the one-year of long-term period.

Methods: Comprehensively characterized stem cell-derived EVs were stored at 4°C for 28 days and −80°C for one year. During given periods, preserved density and differing sizes of EVs were evaluated by nanoparticle tracking analysis (NTA) along with quantitative measurement of variations in total protein and RNA concentrations.

Results: At the 4°C storage condition, concentration and size of EVs were relatively unvarying for 28 days. In terms of total protein and RNA concentrations, about 3–40% of decreasing rates were shown during the first week of period, but rest of the amounts were stably preserved until day 28. At −80°C, EV concentration decreased about 10% from the initial level during the first two weeks, but rest of the amounts were stably preserved for one year. Size of EVs was not changing during the long-term period. In terms of total protein and RNA concentrations, about 50% of decreasing rates were shown during the first two weeks, but rest

of the amounts were stably preserved during the one year of period.

Summary/Conclusion: While a variety of studies are actively ongoing to reach effective cell-free treatments using EVs, the information of EV storage provided by our study would support safe and reliable use of EVs in clinic

Funding: This study was supported by a grant from the Korean Healthcare Technology R&D Project, Ministry of Health & Welfare (HI17C1256) and Basic Science Research Program, the Ministry of Science, ICT and Future Planning (2018M3A9H1023675).

PS06.09

Questioning the purity of the media – extracellular small non-coding RNA contaminants in foetal bovine serum and serum-free media

Bettina I. Mannerström[a], Riku Paananen[b], Ahmed Abu-Shahba[c], Riitta Seppänen-Kaijansinkko[a] and Sippy Kaur[a]

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Introduction: Extracellular vesicles (EVs) behave as paracrine effectors as they are released from cells to deliver signals to other cells. They control a diverse range of biological processes by transferring proteins, lipids and nucleic acids between cells and are secreted by a wide spectrum of cell types and are found in various biological fluids. In the research field of EV research, the use of EV-depleted foetal bovine serum (FBS) for *in vitro* studies is crucial to eliminate the confounding effects of media-derived EVs. The current methods to deplete culture media of EVs are lacking as they do not guarantee an RNA-free preparation.

Methods: In this study we have addressed the RNA contamination issue of EVs in FBS, ultracentrifugation EV-depleted FBS, commercially available EV-depleted FBS, and in our recently developed filtration-based EV-depleted FBS. Commercially available serum-free, xeno-free defined media were also screened for RNA contamination.

Results: Our small non-coding (nc) RNA sequencing data emphasized that all EV-depleted media contained RNA contaminants. Additionally, defined media contained miRNAs and other small RNAs, albeit at a much lower level than in serum preparations. Out of the different FBS preparations studied, our ultrafiltration EV-depleted FBS performed the best in depleting miRNAs. Certain miRNAs, such as miR-122 and miR-203a, proved difficult to remove and were present in all media. As compared to miRNAs, other small RNAs (snRNA, Y RNA, snoRNA and piRNA) were difficult to eliminate from the media.

Summary/Conclusion: Our study showed that even defined media contained trace amounts of small ncRNA. Therefore, in order to screen for baseline RNA contamination in culturing media, RNA sequencing data should be carefully controlled by adding a media sample as a control. This should be a mandatory step before performing cell culture experiments in order to eliminate the confounding effects of media.

Funding: This research was supported by University of Helsinki project funding, Helsinki University Hospital State funding for university-level health research, the Finnish Dental Society Apollonia, Business Finland grant.

PS07: Cellular Uptake of EVs and Membrane Function
Chairs: Quan Lu; Nobuyoshi Kosaka
Location: Level 3, Hall A

15:00–16:00

PS07.01

A tunable system to visualize retrofusion, a major pathway for exosome uptake

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Introduction: Exosomes constitute a crucial mode of intercellular communication, as they can travel through extracellular space to transfer various cellular components from one cell to another. While we understand, to some extent, how exosomal contents can affect recipient cells, the molecular mechanisms governing exosome uptake are yet to be unravelled. Upon encounter with a target cell, exosomes may be internalized and transported to late multivesicular compartments. To avoid imminent degradation in lysosomes, exosomes must escape the endocytic pathway and fuse back to the limiting membrane of multivesicular bodies (MVB) through a process referred to as "back-fusion" or "retrofusion". Within MVBs, retrofusion of intraluminal vesicles (ILV) can notably allow recycling of membrane proteins and also lead to cytoplasmic release of endocytosed viruses. As retrofusion is poorly understood, deciphering its workings would help unfold a major pathway for exosome uptake.
Methods: To enable exploration of this process and ultimately reveal the molecules responsible, we created an inducible system allowing quantification of retrofusion in real time. CD63, a tetraspanin protein localized on both the limiting (LM) and intraluminal membranes (ILM) of late endosomes, was fused to GFP and stably expressed in MelJuso cells, along with two inactive fragments of the tobacco etch virus (TEV) protease. Upon addition of "dimerizer" to the cells, the TEV protease regains activity and cleaves the GFP off of CD63 exposed on the cytosolic side of the LM. A nuclear localization signal then directs this newly liberated GFP to the nucleus. When retrofusion occurs, intraluminal GFP-CD63 repopulates the LM from ILV stores and becomes accessible for TEV protease cleavage, resulting in the increase of nuclear GFP fluorescence over time. Concomitant labelling of acidic vesicles with a fluorescent dye allows for quantification of GFP signal decay specifically from those compartments.
Results: Using this chemically tuneable system, we found that knocking out the lysosomal integral membrane protein Limp2 partially hampers retrofusion, suggesting that Limp2 may be a major player in this process.
Summary/Conclusion: We further aim to identify other proteins implicated in retrofusion in order to propose a suitable mechanistic model.

PS07.02

Uptake of EVs derived from cervical cancer patients with precancerous induces HeLa cell proliferation

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Introduction: Precancerous lesion is defined as early biological effects of cells which occur prior to invasive carcinomas. The lesion is not cancerous and exhibits variations at the cellular and molecular levels in the pathway leading to cancer. Current evidence indicates that extracellular vesicles (EVs) can release from most of the cell types and affect adjacent or distant cells by circulating in all bodily fluids.
Methods: We collected serum of healthy persons and cervical cancer patients with precancerous lesions, stage I, stage II and stage III and then counted concentration and size distribution of the EVs using nanoparticle tracking analysis (NTA). Differential ultracentrifugation incorporated with size exclusion chromatography was used to isolate and purify EVs from pooled serum of each sample groups. Moreover, isolated EVs were investigated their characteristic based on morphology using transmission electron microscope (TEM) and the expression of CD63, CD81, CD9, and Alix protein markers using western blot analysis. Live cell imaging machine was used to monitor uptake of EVs derived from pooled serum of healthy persons or precancerous lesion on HeLa cells.

Results: NTA shows that the concentration of EVs is increased in patients with precancerous lesion and stage I, and declined in the later stages. We also found that EVs isolated from serum of healthy and precancerous group are capable of uptake into the cells within 4 h. Nonetheless, only EVs isolated from precancerous can stimulate HeLa cell proliferation compared to those isolated from healthy and no EVs treatment group.

Summary/Conclusion: This induction would associate with the biomolecules inside of EVs. Our further study is addressing to find out both proteins and regulatory molecules which contribute to cancer progression.

Funding: This work was financially supported by Faculty of Medicine, Prince of Songkhla University and TRF research grant for new scholar.

PS07.03

Optimized protocol for the quantification of amino acid concentrations in exosomes

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Introduction: Exosomes contain parent cell-derived molecules including nucleic acids and metabolites, which are useful as potential biomarkers serving as surrogates of their cells of origin. Accurate quantification of these molecules in exosomes requires to minimize the carryover contamination of residual condition medium (CM) or biological fluids, as they also contain these molecules in high amount. Here, we developed a method for accurate quantification of amino acids (AAs) in exosomes by optimizing pre-analytical sample preparation and applying highly sensitive analytical system. The method enabled us to evaluate the AA profiles of exosomes in comparison with those of CM and cell extracts or biological fluids.

Methods: Exosomes were isolated from CM of human pancreatic cancer cell line, PANC-1, or rat serum by combination of ultrafiltration and ultracentrifugation. AAs were extracted by methanol and analysed by LC-MSMS after pre-column derivatization. AAs concentration and profile were compared among exosomes, CM and parental cells or serum.

Results: Ultrafiltration was introduced to minimize the effect of carryover contamination of residual AAs from CM or serum. A minimal amount of exosomes required for AAs quantification was determined. AA profiles of exosome were different from those of CM and parental cells or serum. In contrast, some changes of intracellular AA concentrations were reflected in exosomes.

Summary/Conclusion: We developed the optimized pre-analytical method for AA quantification in exosomes. This method would be applicable to metabolomics approaches to identify disease biomarkers or surrogate biomarkers for the metabolic status of cells of origin.

PS07.04

Metabolome analysis of pancreatic cancer-derived extracellular vesicles

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Keio university, Tsuruoka, Japan

Introduction: Extracellular vesicles (EVs) are facilitators of cell-to-cell communication. Cancer-derived EVs contribute to cancer progressions such as distant metastasis, angiogenesis and immunosuppression. EVs contain functional cellular components including DNA, mRNA, microRNA and protein. However, metabolome profiling in cancer-derived EVs remains largely unexplored. The purpose of this study is to explain comprehensive metabolite profiling of pancreatic cancer-derived EVs. As a model for studying cancer metabolism, we evaluate the difference between metabolomic profiles in EVs obtained from cancer cells cultured in normoxic or hypoxic conditions.

Methods: Pancreatic cancer cell line Panc-1 was cultivated under normoxic (20% O2) and hypoxic (1% O2) conditions. Cells were sampled using methanol, and EVs were isolated from conditioned medium using ultracentrifugation. The amount of EVs was determined by nanoparticle tracking analysis, and the protein level of the CD9 exosomal marker was measured using enzyme-linked immunosorbent assay (ELISA). Metabolomic analysis was performed by using capillary ion chromatography-mass spectrometry and liquid chromatography-mass spectrometry.

Results: We identified more than 180 kinds of metabolites in pancreatic cancer-derived EVs. Principal component analysis (PCA) of metabolites in EVs showed somewhat differentiated results between normoxia and hypoxia. Further, the metabolite profiles contained in the cells and EVs may be different.

Summary/Conclusion: In conclusion, we optimized the collection protocol of EVs from cultured cell samples for metabolomic analysis. Our results suggested that the metabolic character in EVs may vary that in cells.

Funding: This study was supported by the Japan Society for the Promotion of Science KAKENHI Grants and research funds from the Yamagata Prefecture Government and Tsuruoka City.

PS07.05

Exosomal miR-141-3p regulates osteoblast activity to promote the osteoblastic metastasis of prostate cancer

Yun Ye

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Introduction: Exosomes from cancer cells, which contain microRNA and reach metastasis loci prior to cancer cells, stimulate the formation of a metastatic microenvironment. Previous studies have shown that exosomal miR-141-3p is associated with metastatic prostate cancer (PCa). However, the role and regulatory mechanism of miR-141-3p in the microenvironment of bone metastases require further study.
Methods: In this study, we performed a series of experiments *in vivo* and *in vitro* to determine whether exosomal miR-141-3p from MDA PCa 2b cells regulates osteoblast activity to promote osteoblastic metastasis.
Results: We demonstrate that extracts obtained from cell culture supernatants contained exosomes and that miR-141-3p levels were significantly higher in MDA PCa 2b cell exosomes. Via confocal imaging, numerous MDA PCa 2 bexosomes were observed to enter osteoblasts, and miR-141-3p was transferred to osteoblasts through MDA PCa 2b exosomes *in vitro*. Exosomal miR-141-3p from MDA PCa 2b promoted osteoblast activity and increased osteoprotegerin OPG expression. miR-141-3p suppressed the protein levels of the target gene DLC1, indicating its functional significance in activating the p38MAPK pathway. In animal experiments, exosomal miR-141-3p had bone-target specificity and promoted osteoblast activity. Mice injected with miR-141-3p-mimics exosomes developed apparent osteoblastic bone metastasis.
Summary/Conclusion: Exosomal miR-141-3p from MDA PCa 2b cells promoted osteoblast activity and regulated the microenvironment of bone metastases, which plays an important role in the formation of bone metastases and osteogenesis damage in PCa. Clarifying the specific mechanism of bone metastasis will help generate new possibilities for the treatment of PCa.

PS07.06

Unrevealed mystery of cell dust: extracellular vesicles and tumour derived exosomes

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Introduction: Binding exosomes to their target cells is more likely to be determined by specific interaction(s) of protein(s) enriched in membrane of extracellular vesicles (EVs) including tumour-derived exosomes (TEX) and cellular proteins. Although the exact mechanism of EVs action is not fully understood, several studies reported that TEX can modify tumour microenvironment by transferring non-coding RNAs and miRNA (particularly miRNA-21 and −29) to target cells. Several studies have showed comparative proteomic analyses of exosomes and circulating EVs from different biological fluids validating their respective role as novel biomarkers offering an early, non-invasive method for cancer diagnosis. Protein profiling and detection conducted by label-free quantification will contribute to understanding the contents of healthy and tumour-derived exosomes by identifying their concentration, size distribution, population and composition enabling characterization of peptide peak intensity. Collected data combined into a protein library for early diagnosis will result in the improvement of cancer immunotherapy and new therapeutic targets.
Methods: AB; SEC; IF; NTA;LC-MS; TEM; Flow
Results: According to cell viability assay, exosomes increase cell proliferation after 24 h of treatment for both cell lines: MCF 10 A and A549 for all concentrations of exosomes (0.125, 0.25, 05, 1 mg/mL). Annexin V/ PI assay was used to define the percentage of necrotic and apoptotic cell death after treating cancerous and non-tumorigenic cell lines. Interestingly, the higher percentage of necrotic death (6.45%) was recorded for THP-1 exosomes conditioned with MCF 10 A, while the smallest number of necrotic death was noticed for A549 exosomes condition with the same cell line A549 (1.99%). The TEM analysis showed roughly same size (40–50 nm) of exosomes for all various types of exosomes used in this study (THP-1, MCF 10 A and A549 exosomes).
Summary/Conclusion: In summary, we can conclude that extracellular vesicles (including exosomes) derived from mammalian cell including cancerous and non-

tumorigenic cell line increase cell proliferation. That being said, cancerous cell line secrets quiet a larger amount of extracellular vesicles compared to non-tumorigenic cell line.

Funding: No external fundings.

PS07.07

Surface glycan profiling of extracellular vesicles by lectin array system for biomarker discovery

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Introduction: Extracellular vesicles (EVs) are known as cellular communicators that carry their contents including proteins, lipids and nucleic acids. Since cells handover their biological information to EVs, they can be applicable to cell biomarkers. We showed that glycans on mesenchymal stem cells (MSCs)-derived EVs play important roles in cellular recognition using an evanescent-field fluorescence-assisted lectin array system [1]. Most remarkable feature of this method is that simple, sensitive and real-time detection of surface glycan patterns on intact EVs. In this study, surface glycan profiling on EVs from many types of cells was analysed using the lectin array system.

Methods: EVs were isolated from various kinds of mouse and human cells including cancer cells, undifferentiated and differentiated MSCs, and immune cells by differential ultracentrifugation. Cy3-labelled EVs and their originating cell membranes (CMs) were applied to a glass slide with 45 lectins, and fluorescence intensities were detected using an evanescent-field fluorescence scanner.

Results: Most types of EVs showed higher binding to sialic acids-recognizing lectins and weaker binding to mannose-binding lectin as compared with their originating CMs. Hierarchical clustering analysis and principal component analysis were performed to evaluate whether surface glycans on EVs have their cell specific patterns. The results indicated that glycan profiling of EVs can be used to classify cell types (normal or cancer) and they can be further divided into each type of cancer, MSC sources and cell lineages, indicating that surface glycans on EVs may act as potential biomarkers of cell state.

Summary/Conclusion: In conclusion, a lectin array method is a powerful tool for comprehensively glycan analysis of EVs towards biomarker discovery.

PS07.08

Tomato fruit-derived vesicles: isolation, biocargo characterization and the dissection of different vesicle types

Ramesh Bokka[a];, Gabriella Pocsfalvi[a], Teresa Silvestre[a], Immacolata Fiume[a], Lilla Turiak[b] and Tamás Csizmadia[c]

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Introduction: Plant-derived vesicles are receiving considerable attention due to their potential applications as vectors for the delivery of biologically active substances in the nutraceutical, cosmetic and pharmaceutical fields. Here, in the first time, we report the in depth characterization of micro (MVs) and nanovesicles (NVs) enriched fractions isolated from the pericarp tissue of *Solarium lycopersicum* with the aim to develop a new generation, natural vesicles-based delivery vectors. This includes the setup of a novel GC-MS/MS platform suitable for the characterization of vesicles' metabolites.

Methods: MV and NV fractions were isolated by differential centrifugation. NVs were further purified by sucrose gradient ultracentrifugation method. Isolation of NVs resulted to be troublesome due to the co-purifying pectin substances. Physiochemical properties of the vesicles were analysed by TEM and DLS, while biocargo composition was studied by mass spectrometry-based proteomic and metabolomics workflows. Functional annotation and data mining were performed using Blast2Go software package including InterPro, enzyme codes, KEGG pathways and GOSlim functions.

Results: The isolation method was improved by differential solubilization using 0.1M phosphate 10 mM EDTA buffer pH 8, to keep pectin substances in solution allowing by the efficient purification of NVs. In each sample, approximately 600–800 proteins and approximately 50 metabolites could be identified. A novel method based on GC-MS/MS metabolomic profiling of plant-derived vesicles has been developed.

Summary/Conclusion: Protein biocargo of tomato pericarp tissue-derived vesicles reveals heterogeneous transport and extracellular vesicle subpopulations. More than 340 enzymes comprising 43 antioxidants identified in tomato nanovesicles may count for its

health-promoting effects in the diet, i.e. protection against cancer, maintenance of healthy blood pressure and reduction of blood glucose in diabetic patients.

Funding: This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement number 798576 and FET Open grant agreement number 801338.

PS08: Advances in EV Quantification and Characterization II
Chairs: Cecilia Lässer; Li Min
Location: Level 3, Hall A

15:00–16:00

PS08.01

Taxonomy of individual EVs by nanomechanics

Andrea Ridolfi[a], Marco Brucale[b], Lucia Paolini[c], Costanza Montis[d], Debora Berti[d], Francesco Valle[b] and Paolo Bergese[e]

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Introduction: Probing and understanding the physical properties of individual EVs – as a whole and of their separate components – are fundamental aspects in EV research that still need to be addressed. We will present our latest results regarding the nanomechanical characterization of individual EVs via Atomic Force Microscopy-based Force Spectroscopy (AFM-FS) and discuss their significance and perspectives.

Methods: Our experimental approach entails adsorption of EVs (separated from cell culture media) on inorganic substrates with controlled surface properties. The response of each individual EV to an applied mechanical deformation in physiological buffer is then sampled via liquid AFM-FS. The obtained force curves are finally quantitatively analysed by dedicated models to obtain the EV "nanomechanical fingerprint".

Results: The reversible elastic deformation behaviour of an EV in response to the AFM tip indentation resulted to be the convolution of several characteristics of the EV. We found that the overall apparent stiffness of an intact EV effectively recapitulates its mechanical behaviour. We also found first evidences that this property can be exploited to sort single EVs and that it relates them to other organic envelopes of similar size and composition, such as viruses and synthetic liposomes.

Summary/Conclusion: These results proof that a nanomechanics-based taxonomy might be an important tool for advancing characterization and understanding of EVs at the single vesicle level.

Funding: This research has received funding from the Horizon 2020 Framework Programme under the grant FETOPEN-801,367 evFOUNDRY.

PS08.02

Electrical characterization of individual exosomes secreted from amyloid beta-treated neuroblastoma cells via electrostatic force microscopy

Yeseong Choi[a], Sumi Kim[a], Dae Sung Yoon[b] and Ji Yoon Kang[a]

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Introduction: Exosomes are cell-derived nanovesicles known to provide information about the state of parent cells. Recently, it has been found that the pathogenic amyloid beta oligomers (oAβs), known as a biomarker of Alzheimer's disease (AD), can be propagated between neighbouring neurons through exosomes. At the same time, there is an increasing need to introduce a new technique for the characterization of individual exosomes because of their diversity. In this paper, we used electrostatic force microscopy (EFM) to demonstrate the effect of oAβ on electrical properties of individual exosomes.

Methods: Different concentrations (30, 150, 750 nM) of oAβs were treated to mouse neuroblastoma (N2a) cells, and exosomes were harvested from cell culture media through ultracentrifugation. The electrical properties of exosomes were investigated by using EFM. For EFM experiment, the 10 μL of each exosome solution was deposited on a fresh mica substrate for 15 min, washed in PBS and DW order and dried under pure nitrogen gas.

Results: EFM can visualize the electrostatic force gradient corresponding to the surface potential of single exosomes. The scatter plot resulted from EFM data analysis showed a correlation between the size and the charge of exosomes. Moreover, charge density values, which excludes the influence of size by dividing the charge value by height, decreased by up to four times depending on the concentration when compared with the control (−5.95 μV/nm at control, −9.17, −11.1, −23.85 μV/nm at 30, 150, 750 nM, respectively). It implies that exosomes from oAβ-treated N2a cells have significantly higher negative surface potential than those from untreated N2a cells.

Summary/Conclusion: This paper proposes a new nano-electrical characterization to differentiate neuronal exosomes treated by oAβs from untreated ones. It is

possible to use EFM as imaging and analysis tool for single exosome characterization. Furthermore, it is expected that exosomes associated with AD are isolated from plasma in the diagnosis of AD according to a surface potential of exosome.

PS08.03

Hybrid plasmonic biomaterial nanofilter scaffold for cancer EV diagnostics based on surface-enhanced Raman scattering (SERS)

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Introduction: New analytical approaches are needed that account for the vast molecular heterogeneity of nanoscale extracellular vesicles (EVs). Raman spectroscopy is an attractive technology capable of sensitive molecular fingerprinting of chemical changes associated with disease. Surface-enhanced Raman Spectroscopy (SERS) overcomes the inherent weak nature of spontaneous Raman scattering and is proving to be a promising tool for next-generation clinical diagnostics. The principle of SERS is based on amplification of Raman scattering using metal surfaces that have a nanoscale roughness with features of ~20–200 nm. We introduce an inexpensive and flexible SERS substrate based on a novel biosilica plasmonic nanocomposite that acts as a simultaneous nanofilter and detection platform for sensitive characterization of tumour-associated EVs.

Methods: A porous biosilica scaffold doped with plasmonic silver nanoparticles can be simply and easily prepared on office-grade adhesive tape. This nanocomposite deposition requires no chemical modification of the raw materials. Particles larger than ~100 nm concentrate on the top surface in close proximity to clusters of plasmonic nanoparticles, affording usability as a SERS-based sensing platform.

Results: We tested our platform with dozens of samples of tumour-associated EVs enriched from ovarian cancer patients and healthy controls to demonstrate that SERS imaging can sensitively detect and identify disease profiles. We found enhancement factors of more than 10^8-fold compared to spontaneous Raman signatures. Sensitivity and specificity exceeding 90% was found for human clinical samples using less than 1 µL of minimally processed plasma, all in just a few seconds using a commercial Raman imaging system.

Summary/Conclusion: We introduce a simple plasmonic composite using readily available biomaterials and metallic nanoparticles, and demonstrate its efficacy for label-free sensing of EVs. High chemical specificity afforded by Raman spectroscopy rapidly identified tumour EVs from healthy controls in clinical samples. Our nanocomposites are inexpensive, reusable, stable and suitable for low resource environments, with high potential for translational application of clinical diagnostics using EVs.

Funding: The authors acknowledge funding from the Ovarian Cancer Education and Research Network (OCERN).

PS08.04

Electrochemical quantification of EVs at physiological concentrations

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Introduction: Tumour-derived extracellular vesicles (tdEVs) are promising markers for cancer patient management. An advantage of tdEVs over circulating tumour cells is their higher concentration in patient blood by 3–4 orders of magnitude (~10^3–10^5 tdEVs /ml), giving more robust information while requiring smaller sample sizes. However, their small size and complex composition of blood samples require sensitive and selective detection methods. Here, we report electrochemical detection of tdEVs using a nano-interdigitated electrode array (nIDE) functionalized with cancer-specific antibodies and an antifouling coating. The detection mechanism is based on enzymatic conversion of aminophenyl phosphate (APP) by alkaline phosphatase (ALP) followed by redox cycling of the cleaved substrate, yielding a double signal amplification. The proposed sensing scheme is 10 times more sensitive than state-of-the-art detection approaches, giving a physiologically relevant limit of detection (LOD) of 10 EVs/µl.

Methods: nIDEs (120 nm width, 80 nm spacing, 75 nm height) were functionalized with an amino-undecanethiol monolayer, and reacted with poly(ethylene glycol) diglycidyl ether. Anti-EpCAM antibodies were next immobilized to subsequently capture tdEVs. Anti-EpCAM-alkaline phosphatase conjugates were then introduced to yield ALP-tagged tdEVs. The non-electroactive pAPP was finally used to quantify the ALP concentration.

Results: With increasing tdEV concentration, an increase in redox current was measured, from 0.35 nA for 10 tdEV/µl to 12.5 nA for 10^5 tdEV/µl (avg., $n = 3$). Current is produced by the electroactive

cleavage product of APP, which redox cycles between electrodes. The short migration distance in our nanoelectrode array yielded a factor ~8 improvement compared to micro-electrodes (3 µm width, spacing). As a negative control, the experiment was performed with incubation of platelet derived EVs, whereby the signal did not significantly increase (background current ~0.15 nA).

Summary/Conclusion: A sensitive sensor was developed for the detection of EVs at unprecedented low concentrations. With an LOD of 10 tdEVs/µl and high selectivity towards tdEVs, our platform opens new avenues for screening patient blood samples.

Funding: Funded by NWO Perspectief

PS08.05

The Importance of Orthogonal Techniques in EV Quantification

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Introduction: As EV research matures, so must measurement technologies. Two simple experiments are reported that expose a critical failure mode of Nanoparticle Tracking Analysis (NTA) for quantifying EVs: NTA's small size limit of detection (LOD) depends strongly on the composition of the sample, causing 10,000-fold errors within the EV size range relative to Microfluidic Resistive Pulse Sensing (MRPS) and Tunnelling Electron Microscopy (TEM). Results show orthogonal methods for EV quantification are critical.

Methods: Experiment 1: Three sizes of polystyrene particles – 94, 150 and 208 nm diameters – were measured by NTA and MRPS separately and after mixing in equal parts. The relative concentration accuracy of NTA and MRPS was assessed as a function of size, and the LOD evaluated for each sample.

Experiment 2: The striking implications of Experiment 1 were demonstrated in a real-world sample. Urinary exosomes were measured by NTA, MRPS and the gold standard, Tunnelling Electron Microscopy (TEM). The accuracy of relative concentration measurements was assessed for each method.

Results: Experiment 1: Polystyrene standards were accurately quantified by MRPS: Each component was clearly detected, and the relative concentrations of all were measured to be approximately equal as intended. NTA showed similar results for the separate components. However, NTA was unable to detect the 94 nm particles in the mixture and showed quantification errors at 150 nm diameter.

Experiment 2: MRPS showed the particle size distribution expected: Concentration increased with decreasing particle size with an approximate power-law dependence on diameter reported elsewhere in the literature. MRPS was in excellent agreement with TEM. NTA reported misleading results: A loss of counting efficiency was apparent as high as 200 nm diameter, and led to a 10,000-fold discrepancy by 65 nm. Critically, NTA reported a prominent peak that does not in fact exist.

Summary/Conclusion: These experiments expose a critical failure mode of NTA: Its LOD depends strongly on the composition of the sample, with enormous impact for EV measurements. Critically, a researcher could be severely led astray by the NTA results in isolation, without an orthogonal technique for reference.

PS08.06

Fourier-transform Infrared Spectroscopy (FT-IR) to fingerprint EV subpopulations as a whole

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Introduction: Characterizing EV subpopulations remains a challenge, which up-to-date has been tackled through analysis that assess for a single biochemical or biophysical component of the target subpopulation. However, these approaches may be unsuitable to describe EV subpopulations defined by higher level of heterogeneity. In our contribution, we will discuss how Fourier-transform Infrared Spectroscopy (FT-IR) allows to fingerprint EV subpopulations as a whole, presenting itself as a promising complement/alternative to describe EV subpopulations

Methods: Medium from murine prostate cancer (TRAMP-C2) and skin melanoma (B16) cell lines were processed with serial centrifugation: 800g 30' to enrich large EVs (LEVs), 16,000g 45' to enrich medium EVs (MEVs) and 100,000g for 4 h to enrich small EVs (SEVs). LEVs, MEVs and SEVs were characterized for size, purity and EV markers with Atomic Force Microscopy, colloidal nanoplasmonic assay and

Western Blot, respectively. FT-IR measurements were performed on LEVs, MEVs and SEVs re-suspended in milliQ water and deposited onto a diamond cell. Spectral regions between 3100–2800 cm-1 and 1880–900 cm-1, corresponding to lipids and proteins, respectively, were considered, and processed by Principal Component Analysis (PCA)

Results: PCA was applied to data set of FT-IR spectra (five replicates for each EV subpopulations) collected for TRAMP and B16 cell line and visualized with scores plots. LEVs, MEVs and SEVs resulted grouped separately for both considered cell lines. Moreover, spectra from the same subpopulation, but from different cells are reported in two distinct groups

Summary/Conclusion: EV subpopulations of different sizes and cellular origin are characterized by specific FT-IR fingerprint. This offers a proof of concept that FT-IR could be effectively translated in real scenarios to characterize EVs with different content and origin

Funding: LP acknowledges the BIOMANE grant (University of Brescia) and evFOUNDRY grant (H2020-FETOPEN-2016–2017 – Project ID: 801367) for the financial support

PS08.07=OWP1.07

Exploration of the surface modification of outer membrane vesicles

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Introduction: Introducing bacteria-binding small molecules to the surface of outer membrane vesicles (OMVs) could greatly improve their potential for antimicrobial drug delivery to difficult to treat bacteria. Among the small number of studies on surface modification of OMVs, very few deal with small molecules. The aim of the present study is to evaluate different methods of introducing bacteria-specific targeting moieties to OMVs. We assessed the modification of surface proteins using N-hydroxysuccinimide (NHS) esters, well established for mammalian extracellular vesicles (EVs), cholesterol insertion, mainly applied for liposomes and the novel application of diazo-transfer followed by click-chemistry.

Methods: OMVs were obtained from model Myxobacteria by differential ultracentrifugation (UC) followed by size exclusion chromatography (SEC). For cholesterol insertion and NHS ester-modification, purified OMVs were incubated with either cholesteryl PEG 2000 FITC or sulpho cyanine7 NHS ester. For diazo transfer the pellet after UC was incubated with a diazo transfer agent and the OMVs subsequently conjugated with DBCO-AF594. Unincorporated dye was removed by SEC. Liposomes were composed of DMPC and DPPC in 2:3 molar ratio. Results represent correlated fluorescence intensity and particle number.

Results: Treatment with sulpho cyanine7 NHS ester led to the modification with 547 ± 163 molecules per OMVs, compared to 18 ± 1 for the control using sulpho cyanine7 acid. Cholesterol insertion introduced 4 ± 1 molecules per OMV, compared to 101 ± 23 for liposomes. First results for the diazo-transfer showed 71 dye-molecules per OMV, with 32 for the control.

Summary/conclusion: Of the three methods, NHS ester-modification displayed the highest efficiency, similar to published results for mammalian EVs. In comparison, diazo transfer only yielded ~13% of the dye-molecules per particle. However, there are still many parameters to be optimized for this method, including OMV concentration and incubation period. Cholesterol insertion was unsuccessful for OMVs, probably owing to their membrane structure. In this study, we aim to get important insights into the modification of OMVs for bacterial targeting and EV-surface engineering in general.

Funding: This project was funded by Studienstiftung des Deutschen Volkes and Bundesministerium fuer Bildung und Forschung.

PS08.08=OWP2.01

Identification of common EV markers in plasma using high-resolution flow cytometry

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Introduction: Recent advancements in flow cytometry (FCM) have led to the development of high-resolution FCMs dedicated to the analysis of small particles (hFCM). hFCM studies have predominantly focused on the analysis of EVs expressing phosphatidylserine (PS). PS is enriched in microvesicles (MVs), wherein it is involved in lipid rearrangements responsible for MV budding. While PS also is expressed on exosomes, it is unknown whether it can be used as a universal marker for smaller EVs. In this study, we attempted to characterize proteins enriched in smaller EVs (CD9, CD63,

CD81 and ADAM 10) and the relative co-expression of PS with each of these markers.

Methods: Flow cytometry analysis was performed on an Apogee A60 Micro-PLUS. In brief, platelet-poor plasma (PPP) from healthy individuals was stained with lactadherin-FITC (PS+) and one of several EV surface markers enriched in smaller EVs. To evaluate the precise differences in PS and specific EV marker expression, the analysis was performed twice, (1) triggering on lactadherin and (2) each EV marker (CD9-PE, CD81-PE, CD63-PE, ADAM10-PE), separately. All antibodies were matched with appropriate isotope controls and centrifuged at 17,000*g* for 10 min. prior to antibody labelling. EVs were defined as lactadherin or EV surface marker positive events ≤1000 nm.

Results: Initial results indicate that CD9 is highly expressed on EVs and is not universally associated to PS. Triggering on PS revealed that 34.7% of all events were CD9 positive (CD9+|PS+). Conversely, triggering on CD9 resulted in a 2.1-fold increase in total events, where 17.0% of events were PS+ (CD9+|PS+). Inferring size from silica nanospheres, it appeared that populations containing CD9 (CD9+|PS+ and CD9+|PS-) were smaller (94.4–99.7% <180 nm) compared to populations that did not (PS+|CD9-; 85.6% <180 nm & 95.2% <300 nm). Interestingly, we did not detect CD81, CD63 or ADAM10 on EVs. We hypothesize that this is due to a low abundance of these markers in PPP from healthy individuals.

Summary/conclusion: Our findings demonstrate that hFCM can be used for the characterization of smaller EVs in PPP. Furthermore, we find that CD9+ EVs does not universally express PS. From this point on, we plan to study enrichment of these EV phenotypes following a number of EV purification protocols and determine whether EV isolation enable a more extensive characterization of smaller EVs.

PS08.09=OWP2.02

Software to automate calibration and processing of flow cytometry data in clinical studies

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Introduction: In search of new biomarkers, flow cytometers are used in clinical studies to measure the concentration of specific extracellular vesicles (EVs). Flow cytometers measure light scattering and fluorescence of single EVs in a fluid stream. However, to realize data interpretation and comparison, light scattering and fluorescence signals and the flow rate require calibration. Moreover, flow cytometers generate large datasets. For example, a clinical study involving 60 patients, 30 controls and 8 antibody labels covers 1224 data files, >33 gigabytes of data and >0.3 billion events. To manually calibrate and analyse such a dataset would take days if not weeks and is prone to human mistakes. Therefore, an urgent need exists for software to automate calibration and processing of flow cytometry data.

Methods: We have developed software (MATLAB R2018a) to automatically process multiple .fcs files and (1) relate two scatter signals to the diameter in nm and refractive index (RI) of EVs, (2) express fluorescence signals in terms of molecules of equivalent soluble fluorochrome (MESF), (3) export calibrated channels to new .fcs files, (4) recognize unstable flow rates, (5) determine fluorescence thresholds, (6) apply gates, (7) create PDFs with scatter plots and (8) report statistics. We are using clinical studies to validate and apply the software.

Results: Compared to manual thresholding, automatic thresholding results in a systematic decrease in counts of 10% and a maximum difference of 14% (n = 5). Using a high-end laptop, data processing takes typically a minute or several seconds per .fcs file with or without PDF reporting, respectively. Flow rate monitoring is useful for 61% of the data. The platelet marker CD61 stains 7% of the events with an RI>1.42, which are lipoproteins, and the concentration of these lipoproteins differed 4000-fold between individuals.

Summary/conclusion: We have developed software to automate calibration and processing of flow cytometry data in clinical studies, thereby reducing analyses time, preventing human mistakes and providing new insights. For example, non-specific labelling of antibodies to lipoproteins together with variations in lipoprotein concentrations emphasize the relevance of fasting before venipuncture. Our next step is to extend the software with machine learning.

Funding: NWO-TTW VENI 15924

PS08.10=OWP2.03

Conventional, high-resolution and imaging flow cytometry: potentials, pitfalls and solutions for EV characterization

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Introduction: Flow cytometry (FCM) has long been a preferred method for characterizing EVs, however their small size have limited the applicability of conventional FCM to some extent. Thus, high-resolution and imaging FCMs have been developed but not yet systematically evaluated. The aim of this presentation is to describe the applicability of high-resolution and imaging FCM in the context of EV characterization and the most significant pitfalls potentially influencing data interpretation.
Methods: First, we present a side-by-side comparison of three different cytometry platforms on characterizing EVs from blood plasma regarding sensitivity, resolution and reproducibility: a conventional FCM, a high-resolution FCM and an imaging FCM. Next, we demonstrate how different pitfalls can influence the interpretation of results on the different cytometry platforms. Finally, we propose controls, solutions or workarounds for understanding and limiting the influence of each of these pitfalls.
Results: (1) High-resolution FCM and imaging FCM displayed greater sensitivity and resolution compared to conventional FCM when measuring a mixture of nanospheres. Equally, both methods could detect larger concentrations of specific EV phenotypes than conventional FCM, where imaging FCM outperformed high-resolution FCM. Within day variability (n = 20 aliquots) was similar for conventional and high-resolution FCM, while imaging FCM had a markedly larger variability. Between day variability ($n = 5 \times 5$ aliquots) was similar for all three platforms. (2) The three most substantial pitfalls variably influencing interpretation of results on the three platforms are non-specific binding of labels, antibody aggregates and entities in the sample (i.e. lipoproteins) binding EV-defining dyes. (3) The most important strategies for circumventing these pitfalls are stringent matching, gating and comparison of antibodies and isotype controls, high-speed centrifugation of antibodies and labels prior to staining, and the use and interpretation of stained buffer controls and detergent-treated samples.
Summary/conclusion: High-resolution and imaging FCM hold great potential for EV characterization. However, increased sensitivity also leads to new artefacts and pitfalls. The solutions proposed in this presentation provide useful strategies for circumventing these.

PS08.11=OWP2.04

Convolutional neural networks for classification of tumour derived extracellular vesicles

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Introduction: Raman spectroscopy probes molecular vibration and thus reveals chemical information of a sample without labelling. This optical technique can be used to study the chemical composition of diverse EVs subtypes. EVs have a complex chemical structure and heterogeneous nature so that we need a smart way to analyse/classify the obtained Raman spectra. Machine learning (ML) can be a solution for this problem. ML is a widely used strategy in the field of computer vision. It is used for recognizing patterns and images as well as classifying data. In this research, we applied ML to classify the EVs' Raman spectra.
Methods: With Raman optical tweezers, we obtained Raman spectra from four EV subtypes – red blood cell, platelet, PC3 and LNCaP – derived EVs. To classify them by their origin, we used a convolutional neural network (CNN). We adapted the CNN to one dimensional spectral data for this application.
The ML algorithm is a data hungry model. The model requires a lot of training data for accurate prediction. To further increase our substantial dataset, we performed data augmentation by adding randomly generated Gaussian white noise.
The model has three convolutional layers and fully connected layers with five hidden layers. The Leaky rectified linear unit and the hyperbolic tangent are used as activation functions for the convolutional layer and fully connected layer, respectively.
Results: In previous research, we classified EV Raman spectra using principal component analysis (PCA). PCA was not able to classify raw Raman data, but it can classify preprocessed data. CNN can classify both raw and preprocessed data with an accuracy of 93% or higher. It allows to skip the data preprocessing and avoids artefacts and (unintentional) data biasing by data processing.
Summary/conclusion: We performed Raman experiments on four different EV subtypes. Because of its complexity, we applied a machine learning technique to classify EV spectra by their cellular origin. As a result of this approach, we were able to classify EVs by cellular origin with a classification accuracy of 93%.

Funding: This work is part of the research program [Cancer-ID] with project number [14197] which is financed by the Netherlands Organization for Scientific Research (NWO).

PS08.12=OWP2.05

Microfluidic electrochemical aptasensor for detection of breast cancer-derived exosomes in biofluids

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Yonsei University, Seoul, Republic of Korea

Introduction: Exosomes are nanosized extracellular vesicles, which are emerging as potential non-invasive biomarkers for early diagnosis of cancer. However, the small size and heterogeneity of the exosomes remain significant challenges to their quantification in the biofluids. In the present research, a microfluidic electrochemical biosensing system (MEBS) is introduced to detect ultra-low levels of breast cancer cell-derived exosomes (BCE).

Methods: Fabrication procedure of MEBS comprises three main steps: first, biosensing surface was prepared by immobilizing EPCAM binding aptamer (EBA) on a nanostructured carbon electrode. The nanostructured surface (NS) consists of 2D nanomaterials including MoS_2 nano-sheets, graphene nano-platelets and a well-ordered layer of electrodeposited gold nanoparticles. The NS was well characterized with FESEM and EDX. FESEM analysis showed a well-ordered gold nano-structuring for 50 nM of gold solution. Furthermore, EDAX analysis confirmed >60% coverage of gold nanoparticles on nano-structured surface compared to bare carbon electrode. At the second step, a herringbone structured microfluidic channel, which is able to enrich BCE was designed and fabricated. Finally, microfluidic channel was integrated to biosensing surface. Different concentrations of exosome solutions was introduced and enriched to biosensing surface (SPCE/NS/GNP/EBA) using microchannel. After capturing BCEs on the sensing surface a secondary aptamer labelled with silver nanoparticles (SNPs) as redox reporter was introduced to the sensing surface.

Results: Direct electro-oxidation of SNPs was monitored as analytical signal. The unique design of microchannel in combining with high-specific interaction between BCE and EBA provided a high-sensitive detection of BCE as low as ~100 exosomes/µl.

Summary/conclusion: The unique design of MEBS provides a highly sensitive accurate platform for detection of ultra-low levels of cancer-derived exosomes. This tool holds great potential for early cancer diagnosis in clinical applications.

PS08.13=OWP2.06

A software suite allowing standardized analysis and reporting of fluorescent and scatter measurements from flow cytometers

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Introduction: Single vesicle analysis using flow cytometry is an extremely powerful technique to allow identification of unique proteins in biological samples, as well as enumerating the changes in concentrations. While small particle analysis (for viruses and large microparticles) using flow cytometry has been conducted for several decades, there is no comprehensive method for standardization of such studies. Therefore, we developed a suite of flow cytometry post-acquisition analysis software (FCMPASS) tools that enable the conversion of scatter and fluorescent axes to standardized units using appropriate controls, writing standardized units to .fcs files for sharing upon publication with open repositories, and exporting templates of obtained data.

Methods: Standalone software packages for scatter and fluorescent standardization were built using MATLAB. The scatter software is based upon Mie modelling and is capable of predicting the optical collection angle of the instrumentation and reporting the Mie modelling criteria in a standardized way, making it possible to reproduce the models and flow cytometry settings. Fluorescent standardization data uses least-squares linear regression to enable conversions of arbitrary unit scales to molecules of equivalent soluble fluorophore (MESF) using MESF calibration beads.

Results: The FCMPASS software converts arbitrary fluorescence units to MESF units and writes them to data files for clearer reporting and sharing of data. FCMPASS also converts arbitrary scatter units to a measurement of scattering cross-section using modelling software that predicts the collection angle of the instruments and normalizes the data automatically.

Summary/conclusion: Utilization of our FCMPASS software can help the EV flow cytometry more easily implement standardization into their experimental analysis and the use of the output templates can make reporting more consistent. While currently available MESF controls can be further optimized for small particles, we believe their utilization along with the other controls and can bring a new era to the reporting of EV research using flow cytometry. This will be

particularly useful for future comparison and validation of translational studies and will enable better understanding and utilization of EVs across a broad range of disciplines.

PS08.14

The effect of antibody binding on the zeta potential of extracellular vesicles secreted by cultured human choriocarcinoma cells

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Introduction: Research on extracellular vesicles (EVs), which include exosomes and microvesicles, has witnessed an exponential increase in the past decade. EVs are membrane-derived vesicles, which play vital role in transporting functional molecules to nearby or distant cells, thus being involved in the intercellular communications. Developing a reliable and quantitative method for confirming a nanoparticle as an EV is still challenging. Nanoparticles carry a net surface charge due to the nature of their surface molecules. We have hypothesized that EVs, which normally carry a negative zeta potential (ZP), can be identified by the change of net surface charge when bound to EV-specific antibodies.

Methods: ZP measurements were performed on EVs collected from the conditioned medium of human choriocarcinoma (JAr) cells grown in EV-depleted media. EVs were purified using size exclusion chromatography. EV populations were incubated with EV surface membrane-specific antibodies and the change in the electrokinetic mobility upon the binding of surface EV proteome with an antibody was measured using nanoparticle tracking analysis (Zetaview; Particlemetrix, Inning, Germany).

Results: The mean+SEM ZP was −22.1 ± 0.8 mV and −20.5 ± 0.8 mV for non-treated JAr EVs and immunoglobulin G isotype antibody (control)-treated EVs, respectively, indicating the absence of influence of nonspecific binding. Whereas the ZP distribution of EVs incubated with surface exosomal marker antibodies showed a significant positive shift in the measured values compared to EVs incubated with control antibody. The mean+SEM ZP values of EVs bound with CD63 and CD81 were 17.2 ± 1.1 mV and −17.8 ± 0.9 mV respectively (N = 3 biological replicates of minimum 1000 particles measured in each replicate). Western blot analysis showed particles carrying EV-specific surface markers. Furthermore, we investigated the other factors that may have a potential effect on the changes in EV’s electrokinetic mobility such as the concentration of particles and concentration of the antibody.

Summary/conclusion: The measured antibody-specific changes in ZP values provide an insight into the nature of the nanoparticle surface antigens in a biological sample. ZP measurement is a simple, cost-effective and reliable method for profiling EV surface composition.

PS09: EV Cancer Pathogenesis
Chairs: Marta Prieto Vila; Judy Yam
Location: Level 3, Hall A

15:00–16:00

PS09.01

Extracellular vesicles secreted from ganglioside GD3-expressing cancer cell lines contain high levels of integrins: Roles of lipid rafts

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Introduction: Cancer-associated glycosphingolipids have been utilized as tumour markers and targets of cancer therapy. We have analysed functions of gangliosides in cancers, and reported that cancer-associated gangliosides enhance malignant properties of cells by forming complexes with various membrane molecules. In this study, we have examined contents of extracellular vesicles (ECVs) secreted from ganglioside GD3-expressing cancer cells to investigate roles of gangliosides in the regulation of ECVs, leading to the induction of cancer microenvironments and metastasis.
Methods: GD3-positive cells as well as GD3-negative control cells were established by transfection of GD3 synthase (ST8SIA1) cDNA into melanoma, glioma and small cell lung cancer (SCLC) cell lines. ECVs were collected from culture supernatants by repeated ultracentrifugation. Contents in ECVs were analysed by Western blotting. Roles of lipid rafts were analysed by treating cells with 1 mM methyl β-cyclodextrin.
Results: In ECVs from GD3-positive melanoma cells, GD3 and ST8SIA1 mRNA were detected in TLC and by RT-qPCR, respectively. In Western blotting, increased levels of integrin families were detected in ECVs from GD3-positive melanoma cells compared with those from GD3-negative cells. Similar increase of integrins was also found in glioma and SCLC cells. This was contrastive with integrin levels in cell lysates from GD3-positive and – negative cells, showing almost equivalent levels of integrins regardless of GD3 expression. Particularly in melanoma cells, levels of integrin α2, β1 and β2 showed marked increase in GD3-positive cell-derived ECVs. Treatment of GD3-positive melanoma cells by 1 mM methyl β-cyclodextrin resulted in marked reduction of exosomes and TSG101 in them.
Summary/Conclusion: GD3 expression in cancer cells resulted in increased levels of integrins in ECVs, suggesting that GD3 and integrins play roles in the malignant properties of cancers by forming molecular complexes on ECVs. Lipid rafts may play roles as sites for the complex formation.
Funding: Grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan

PS09.02

Amniotic Epithelial Exosomes Result In Reversal of Epithelial to Mesenchymal Transition in Hepatocellular Carcinoma Cell Lines

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Introduction: Mesenchymal type hepatocellular carcinoma (HCC) with epithelial to mesenchymal transition (EMT) constitutes the most aggressive HCC. Our work has shown that exosomes from amniotic epithelial cells (AECs), an intriguing cell from the epiblast which can switch between epithelial and mesenchymal phenotype, contain a myriad of growth and signalling factors that regulate cell differentiation and has immunomodulatory and antiproliferative properties. We hypothesize that modulation of HCC differentiation into more differentiated epithelial phenotype via amniotic epithelial cell exosomes will abrogate aggressive biology.
Methods: Size exclusion chromatography via the use of qEV columns was used to separate AEC media into exosome (less than 100 nm) and non-exosome fractions (more than 100 nm). Using the MACSPlex exosome kit, we showed the abundant expression of CD63, CD9 and CD81 in these AEC exosomes. HUH-7, SK Hep-1 and HLF cell lines were seeded into plates treated with exosomes, non-exosome fractions and control daily. Proliferation and migration were assessed over 72 h by Alamar blue, Glo and wound healing assays.

Immunofluorescence for vimentin, E cadherin, KDR and EPCAM were performed to assess for epithelial to mesenchymal transition (EMT).
Results: The proliferation of all three cell lines were significantly reduced in the exosome and non-exosome arms compared with control, on both Alamar Blue stain and Glo assay (all $p < 0.05$). Wound healing was reduced significantly in the exosome arm vs. control in Sk-Hep1 and HLF ($p = 0.016$ and 0.004, respectively), but not in HUH-7 ($p = 0.156$).

On immunofluorescence, there was upregulation of the epithelial marker E cadherin in the exosome and non-exosome arms in SK-Hep1 and HUH7, but it was not expressed in the control arm. E cadherin was upregulated in the cells treated with exosomes compared to non-exosomes in SK-Hep1 and HUH7. There was downregulation of the mesenchymal marker vimentin in the HLF cells treated with exosomes and non-exosomes as compared to control.
Summary/Conclusion: Exosomes have the ability to modulate HCC tumour biology, possibly by pushing HCC cell lines into mesenchymal epithelial transition to become less proliferative and motile.

PS09.04

Extracellular vesicles miRNA in mediating EGFR-TKI sensitivity in heterogeneous EGFR-mutant NSCLC

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Introduction: Tumour heterogeneity has impacts on targeted drug resistance. At lung cancer, the discordance rates of EGFR mutation implying tumour heterogeneity in metachronous and synchronous settings were 14.3% and 9.1%, respectively. Extracellular vesicles (EVs) serve as the transporter of bioactive molecules between cells and become one of the major mechanisms contributing intratumoural heterogeneity via transferring genetic information. Since most patients harbouring EGFR mutation showed excellent response, we hypothesized that EVs mediate the cross-talk between EGFR mutant cell and EGFR wild type cell contributing the change of sensitivity of EGFR wild type cell to EGFR-TKI in heterogeneous NSCLC
Methods: We used ultrafiltration (UF) method to isolate the EV. To mimic tumour heterogeneity, we next tested the significance of EV on EGFRTKI sensitivity of CL1-5 (EGFR-wild) in co-culture system with PC9 (EGFR-mutant) pretreatment with or without GW4869. To further evaluate the role of EV in gefitinib resistance, we harvested EV from PC9 cells and evaluated their effect on gefitinib sensitivity of CL1-5 in orthopedic animal model. We further compared the EV miRNAs from PC9 to those from CL1-5 and identified a panel of discriminative miRNAs.
Results: The CL1-5 uptake of PKH26 labelled exosomes derived from PC9 cell can be recorded by time-lapse microscope. And the EGFRDel19 DNA and specific protein can be detected in recipient wild-type EGFR cells by digital PCR and Western blotting respectively. We demonstrated that wild-type EGFR lung cancer cell became sensitive to EGFR-TKI after co-culture with PC9 cell for 48 h and then subjected to gefitinib for 72 h. However, the pretreatment with GW4869 for 48 h reversed the sensitivity to EGFR-TKI in co-culture system with PC9. In CL1-5 animal model, neither gefitinib nor exosome treatment alone inhibited tumour growth compared to control group. Only combination treatment with exosome and gefitinib delayed tumour growth. Some miRNA among the panel such as miR-200 family have been identified associated with resistance to EGFR-TKI
Summary/Conclusion: Our study proposed that in heterogeneous EGFR-mutant NSCLC, tumour cells share biomolecules such as through local and systemic transfer of EVs, which may affect cell sensitivity.
Funding: MOST-107-2314-B-006 –069 -

PS09.05

Senescent cells-derived extracellular vesicles repress tumour growth by transferring miR-127-3p and miR-134-5p.

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Introduction: The mechanism called cellular senescence avoids tumourigenesis by arresting DNA-damaged cells growth. The microRNAs are about 20-nt non-coding RNAs. MiRNAs complementary bind to target mRNA and suppress their translations and/or stabilities. Cellular miRNAs play important roles in cellular senescence induction, and termed as senescence associated miRNAs. MicroRNAs are transferred by extracellular vesicles (EVs), and regulate phenotypes of recipient cells. However, the roles of EV-miRNAs secreted from senescent cells are still unclear. In this study, we examined

whether EVs and EV-miRNAs secreted from senescent cells regulate cancer cell's activities.

Methods: The normal fibroblast TIG-3 was continuously cultured to establish replicative senescent cells. EVs were collected by ultracentrifugation. Particle numbers and their size distributions were analysed by a tunable resistive pulse sensing instrument (qNano; IZON Science). The expressions of exosomal marker proteins were analysed by western blot. MicroRNA expression profiles were analysed by next-generation sequencing. MicroRNA and mRNA expressions were quantified by quantitative reverse transcription polymerase chain reaction.

Results: EV secretion was elaborated in replicative senescent TIG-3 cells. Senescent cell-derived EVs (S-EVs) treatment repressed growth of breast cancer cell line MDA-MB-231. The expression of miR-127-3p and miR-134-5p were enriched in S-EVs. Mir-127-3p and miR-134-5p expressions were increased in S-EVs treated cancer cells. Growth arrest activity of S-EVs was inhibited by pretreatment of LNA-miRNA inhibitor for miR-127-3p and miR-134-5p in MDA-MB-231.

Summary/Conclusion: Senescence cell-derived extracellular vesicles inhibited tumour growth by transferring miR-127-3p and miR-134-5p.

PS09.06

Potential roles of cancer derived extracellular vesicles in lung cancer metastasis and progression

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Introduction: Cells release different types of nanometre sized extracellular vesicles (EVs) of endosomal and plasma membrane origin consisting into the extracellular environment to mediate intercellular communication. EVs have been shown to play important roles in many diseases including tumour. However, the role of EVs in lung cancer is still not fully understood. In this study, we tried to find out the biological functions of EVs in lung cancer.

Methods: EVs were isolated from culture supernatants, serum, and malignant pleural effusion (MPE) using ultra-centrifugation (UC) and ultra-filtration (UF) and then evaluated by TEM, cryo-EM, and Nanosight. The biological functions of EVs were analysed in both in vitro cell line model and in vivo animal model.

Results: EVs were isolated from culture supernatants from both cell lines and ex vivo cultured cancer associated cells, and clinical biofluids using the classical ultra-centrifugation (UC) method and alternative ultra-filtration (UF) method. The EVs could be uptake by lung cancer cells and trigger oncogenic signals such as Stat3 and Akt. Previously, we have shown that IL-6/Stat3/tissue factor (TF)/VEGF pathway plays an important role in lung cancer angiogenesis and metastasis. Here, we showed that EVs from lung cancer samples carried high level of VEGF and TF and triggered vascular permeability changes in both in vitro and in vivo models.

Summary/Conclusion: Using the UC as well as the UF methods, we isolated EVs not only from culture supernatants but also lung cancer associated clinical samples and showed that the EVs triggered oncogenic signals in an autocrine/paracrine fashion and increased vascular permeability. These results may help the understanding of potential roles of cancer derived extracellular vesicles in lung cancer metastasis and progression.

Funding: This work was financially supported by the Centre of Applied Nanomedicine from The Featured Areas Research Centre Program within the framework of the Higher Education Sprout Project by the Ministry of Education in Taiwan, MOHW 106-TDU-B-211–144004 and MOHW 105-TDU-B-211–133016 from the Ministry of Health and Welfare in Taiwan, MOST 106–2314-B-006–040-MY2, and MOST 104-2314-B-006-046-MY3 from the Ministry of Science and Technology in Taiwan.

PS09.07

Whole transcriptome and miRNome profiling of plasma-derived extracellular vesicles cargo in haematological malignancies.

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Introduction: Extracellular vesicles (EVs) role in patients with haematological malignancies has not been investigate as extensively as in solid cancers. In this study, the overall composition of RNA species content of plasma derived EVs isolated from lymphoid and myeloid malignancies (B-cell chronic lymphocytic – CLL, acute myeloid – AML, acute lymphoid – ALL leukemia, monoclonal B-cell lymphocytosis – MBL, myelodisplastic syndrome – MDS, myeloproliferative neoplasms – MPN) was investigated.

Methods: Participants gave written informed consent in accordance with the Declaration of Helsinki. EVs were isolated with ExoquickTM (System Biosciences) from

plasma collected from patients and then analysed with Nanosight. Whole transcriptome (WTS) and small RNA sequencing were performed respectively on 123 and 256 samples. TruSeq stranded mRNA library preparation kit (Illumina) was used to detect coding and long non-coding RNAs. Small RNA libraries were prepared using the NebNext kit (NEB). Differential expression (DE) analysis of RNA species was done with EdgeR Bioconductor package (ANOVA-like) and DESeq2 implemented in docker4-seq package using as reference the expression values detected in HD.

Results: The analysed EVs have size ranging between 80 and 250 nm. WTS generated, on average, more than 10 million mapped reads/samples. The RNA cargo was mainly composed of protein coding genes (95%), and the remaining fraction by lincRNAs and processed pseudogenes. 48 RNAs were detected as DE comparing diseases to HD. Among them 14 were mitochondrial pseudogenes overexpressed in all diseases with respect to HD and their expression is higher in chronic versus acute diseases. Small-RNA seq generated at least 100,000 mapped reads/samples. Sets of miRNAs able discriminate each disease from HD were also detected. Further, analysis to detect disease-specific and disease-predictive signatures are in progress.

Summary/Conclusion: This study gives an overview of plasma derived EVs RNAs cargo in haematological diseases. The analysis of the common/unique RNA biotypes and the evaluation of their expression levels among samples, can guide the identification of patients' stratification markers. Moreover, this study provides a collection of EVs-associated RNAs/miRNAs to be used as reference in different applications in liquid biopsy research.

Funding: FP7 NGS-PTL European grant

PS09.08

The mechanism of non-metastatic contagious carcinogenesis

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Introduction:

Background: Head and neck squamous cell carcinoma (HNSCC) has a high recurrence and metastatic rate with an unknown mechanism of cancer spread. Tumour inflammation is the most critical processes of cancer onset, growth and metastasis. By inflammation, tumour cells can establish an immunosuppressive microenvironment to induce cancer progression.

Hypothesis: We hypothesize that the release of extracellular vesicles (EVs) by tumour endothelial cells (TEC) induce reprogramming of immune cells as well as stromal cells to create an immunosuppressive microenvironment that favour tumour spread. We call this mechanism as non-metastatic contagious carcinogenesis.

Methods: EVs were collected from primary HNSCC-derived endothelial cells (TEC-EVs) and were used for stimulation of peripheral blood mononuclear cells (PBMC) and primary adipose mesenchymal stem cells (ASCs). Regulation of ASC gene expression was investigated by RNA sequencing and protein array. PBMC stimulated with TEC-EVs were analysed by ELISA and FACS. The effect of ASCs or PBMC, treated with TEC-EVs, we demonstrated on tumour cells using various *in vitro* assays, such as invasion, adhesion or proliferation.

Results: We found and confirmed that TEC-EVs were able to change ASC inflammatory gene expression within 24–48 h. TEC-EVs were also able to enhance the secretion of TGFb1 and IL-10 by PBMC and to increase T regulatory cell (Treg) expansion. TEC-EV carries specific proteins and RNAs relevant for Treg differentiation and immune suppression. ASCs and PBMC, treated with TEC-EVs, enhanced proliferation of tumour cells, their adhesion, and invasion, therefore driving non-metastatic cancer spread.

Summary/Conclusion: Conclusions. These data indicate that TEC-EVs are a mechanism of non-metastatic contagious carcinogenesis that regulates tumour microenvironment and reprogrammes immune cells to sustain tumour growth and progression.

Funding: NIH fund R21DE025398, Grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) projects IG 2015.16973 and IG 2015.17630

PS09.09

Exosomes from mitotic slippage-induced senescent cells stimulate inflammatory response

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Introduction: Microtubule-targeting drugs are the most-commonly used first-line chemotherapy. We previously showed nocadazole treatment can lead to paracrine pro-tumorigenic effects via mitotic slippage-induced senescence. Senescent cells exosomes, which

role in non-cell autonomous cell-cell communication. The aim of this study was to decripher effect of exosomes released from senescent-inflammatory breast cancer cells post-slippage on recipient normal breast cells.
Methods: MDA-MB-231 and MCF-10A breast cancer cell lines treated with Noc (100 ng/μl) for 72 h. Conditioned media (CM) was prepared after Noc and DMSO treatment by incubating cells in growth media containing exosome-depleted FBS for 72 h. CM was then collected and centrifuged at 500 ×*g* 10 min, 2000 ×*g* 30 min and 15,000 ×*g* 30 min at 4°C to remove cells and large debris. Supernatant was filtered, exosomes pelleted at 120,000 ×*g*, 2 h, 4°C, washed with PBS, centrifugation at 100,000 ×*g*,1 h, 4°C. Exosomes were dissolved in PBS for whole exosome experiments or processed for total RNA, miRNA and protein isolation for microRNA profiling, RNA-seq and mass spec.
Results: Mitotic-slippage-induced senescent (MIS) cells activate NFκB pathway and increase exosome production, assessed via immunoblots of cytoplasmic and nuclear protein fraction, and IF for p65 localization. We characterized exosomal proteins using TMT labelling and detected significant upregulation of caveolin-1 in Noc treated exosomes. Exosomal microRNA also showed significant upregulation of inflammatory pathway-related genes upon Noc-treatment. Exosomes were transferred from MDA-MB-231 cells after Noc treatment to the recipient MCF-10A cells. Uptake of MIS-derived exosomes resulted in transfer of NFκB response in recipient cells.
Summary/Conclusion: Noc treatment leads to MIS and inflammation in MDA-MB-231 cells. Exosomes released from senescent-inflammatory breast cancer cells contribute to transfer of soluble factors which activate inflammatory pathway in recipient cells. Hence, senescence-induced exosomes can transfer therapy-induced immune signalling via non-cell autonomous mechanisms.
Funding: National Research Foundation Fellowship Singapore MOE AcRF Tier 2015-T1-002-046-01.

PS09.10

Extracellular vesicles from breast cancer cells deliver microRNA-125b to activate cancer-associated fibroblasts

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Introduction: Extracellular vesicles (EVs) are often released by tumour cells for intercellular communication with other cell types in the tumour niches. However, it is unclear which cell type is the most frequent recipient of tumour EVs *in vivo*.
Methods: To analyse the cell types taking up EVs from tumour cells, we created breast cancer cell lines secreting fluorescent EVs, with CD63-GFP fusion protein or with surface mCherry. The cells were implanted in the mouse mammary fat pad or tail vein and the uptake of EVs were analysed in different cell populations of the tumours and the lungs using FACS. We then purified EVs from breast cancer cells using ultracentrifugation and profiled miRNAs using sequencing. The abundance of miR-125b was validated in size exclusion chromatography -purified EVs. The function of miR-125b was analysed by knockdown or overexpression experiments.
Results: We found that fluorescent EVs from tumour cells are taken up most robustly by fibroblasts in the tumours or the metastatic lungs. Our RNA sequencing data revealed that miR-125b is one of the most abundant microRNAs in the EVs from mouse 4T1 and 4TO7 cells. Treatment with 4T1 EVs promotes fibroblast activation in isogenic 4TO7 tumours. This is rescued by knocking down miR-125b in 4T1 EVs; therefore, miR-125b transfer by EVs is responsible for the fibroblast activation. Similarly, we found that miR-125b is abundant in EVs from human breast cancer cells. The uptake of EVs from human breast cancer cells increases cellular levels of miR-125b in the resident fibroblasts hence upregulates several markers of cancer-associated fibroblasts in vivo. miR-125b overexpression also upregulates alpha-SMA and promotes invasion of isolated fibroblasts in vitro. We further identified Tp53 and Tp53inp1 as the targets of miR-125b that are responsible for the phenotype.
Summary/Conclusion: In summary, our study shows that the delivery of miR-125b in EVs from breast cancer cells to resident fibroblasts promotes the development of cancer-associated fibroblasts in the tumour microenvironment.
Funding: This study is supported by City University of Hong Kong (grant 9610343, 9667133 and 7200475), the Hong Kong Health and Medical Research Fund (03141186), the Hong Kong Research Grants Council (21106616) and the National Natural Science Foundation of China (81602514 and 81773246).

PS09.11

Carnitine palmitoyltransferase 1 regulates proliferation of prostate cancer cells under hypoxia via extracellular vesicles-mediated removal of oxidized proteins

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Introduction: Prostate cancer (PCa) is the most common cancer in men with 164,690 new cases and 29,430 deaths estimated in the USA in 2018. Studies have shown that hypoxia determines PCa aggressiveness, and poor outcome. We have recently reported that increased extracellular vesicle (EV) secretion under hypoxia promotes survival of aggressive PCa cells; here, we focused on underlying molecular mechanism/s.

Methods: PCa cells were cultured under normoxia (~21% O2) or hypoxia (1% O2), and EVs (exosomes) isolated from the conditioned media by ultracentrifugation. β-oxidation was measured using a novel PET tracer (18[F]Fluoro-4-thia-oleate), reactive oxygen species (ROS) levels measured by DCF-DA staining, and oxidized proteins in PCa cells and EVs were measured using an oxidative stress sensor (BP1:biotin-1,3-cyclopentanedione) followed by immunoblotting and mass spectrometry (MS). CPT1 knock-down and overexpressing PCa cells were generated using lentiviral particles.

Results: We identified PCa cell lines (E006AA-hT, MDA PCa 2b, 22Rv1 and WFCB17) with higher proliferation under hypoxia compared to normoxia. These cells showed increased lipid uptake and β-oxidation under hypoxia. CPT1 is the main regulator of β-oxidation, and CPT1 knock-down in PCa cells significantly reduced the viability, clonogenicity and stemness under hypoxia; while CPT1 overexpression increased the PCa cells proliferation, clonogenicity and stemness. Both hypoxia and β-oxidation are known to promote oxidative stress, and we also observed high ROS levels in PCa cells under hypoxia. We also observed higher amount of oxidized proteins in hypoxic PCa cells, measured by BP1 labelling and immunoblotting. MS analyses identified the signature of oxidized proteins that were altered in PCa cells under hypoxia. Interestingly, PCa cells proliferating under hypoxia secreted increased concentration of EVs, loaded with high amount of oxidized proteins; while treatment with either exosomes biogenesis inhibitors (GW4869 and DMA) or antioxidants (N-acetylcysteine and ascorbic acid) strongly reduced the growth of PCa cells under hypoxia.

Summary/Conclusion: PCa cells could proliferate under hypoxia through CPT1-mediated increased β-oxidation and via managing high intracellular oxidative stress through EV-mediated removal of oxidized proteins.

PS10: EV Cancer Pathogenesis II
Chairs: Hiroshi Ageta; Ming Jer Tang
Location: Level 3, Hall A

15:00–16:00

PS10.01

Prostate cancer cells promote bone metastasis through extracellular vesicles

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Introduction: Bone metastasis (BM) is one of the major concerns that causes skeletal-related events and increases mortality in prostate cancer (PCa) patients. Vicious cycle paradigm has been proposed to describe how PCa cells educate osteoblasts and osteoclasts (OCs) to benefit the survival and growth of the PCa cells in the metastatic site. However, the underlying mechanisms of BM in PCa remain obscure. Here, we show that extracellular vesicles (EVs) from PCa cells (PCa-EVs) are involved in the vicious cycle, and contribute to the progression of BM.

Methods: PCa-EVs and normal prostatic epithelial cell (NPE)-derived EVs (NPE-EVs) were isolated by ultracentrifugation and evaluated their effect on OC differentiation by Tartrate-resistant acid phosphatase (TRAP) stain. PCa-EVs and NPE-EVs were analyzed using LC-MS/MS to identify candidate proteins which promote OC differentiation. Then, a small-scale screening was conducted using siRNA in PCa cells to determine proteins essential for osteoclastogenesis. The expression level of the specific molecule on EVs was evaluated in clinical samples.

Results: We found that PCa-EVs promoted OC differentiation in the presence of RANKL. In addition, RNA sequence analyses confirmed the drastic change of gene expression essential for osteoclastogenesis in OC precursors. Moreover, we found a specific molecule on EVs which promote OC differentiation. Elimination of the molecule on PCa-EVs led to the attenuation of OC differentiation. In addition, overexpression of this molecule promoted OC differentiation. Finally, we found the molecule on EVs was specifically detected in plasma-derived exosomes from PCa patients with bone metastasis compared to non-metastatic PCa patients.

Summary/Conclusion: PCa-EVs synergistically activate osteoclastogenesis with RANKL. PCa-EVs will be the novel diagnostic and therapeutic target for BM in PCa, leading the great improvement of quality of life in PCa patients.

PS10.02

Novel Exosomal miRNAs-891-5p as an Indicator of Chemoresistance in Ovarian Cancer

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Introduction: Ovarian cancer patients usually have a poor prognosis and low five year's survival rate because it predominantly presents at late stages of the disease. New approaches are required to develop more effective early detection strategies and real-time response monitoring to the available treatments. Thus, this study aimed to identify an exosomal signature which can be used to determine a patient's response to the chemotherapy.

Methods: A panel of ovarian cancer cell lines were used in this study. Cell migration, proliferation and apoptosis in response to different concentrations of carboplatin (0-100 µM) were evaluated using a real-time monitoring system (Incucyte). The miRNA profile was determined using TruSeq® SmallRNA Library (Illumina). Hierarchical clustering and principal component analysis (PCA) were used for multi-omics analyses. Subsequently, candidate miRNAs inducing chemoresistance was confirmed in cells and their exosomes. Candidate miRNAs (mimic) were incubated on sensitive ovarian cancer cells (CAOV-3) and cells response to carboplatin was determined. Finally, a set

of miRNAs were validated in circulating exosomes obtained from a small cohort of patients who experience cancer relapse.

Results: The migration capacity of these cells were associated with cell apoptosis in response to carboplatin with EC50 (concentration of a drug that gives half-maximal response) of 12.1 ± 2.6, 9.4 ± 2.2, 4.4 ± 1.5, 4.1 ± 1.6, 4.0 ± 1.9, 2.8 ± 0.9, 1.5 ± 0.6, 0.9 ± 0.2 and 0.7 ± 0.1 for HEY, SKOV-3, OVACR-429, OV90, OVTOKO, OVCAR-420, OVCAR-3, CAOV-3 and TOVII-2D, respectively. In contrast, the proliferation of these cells was inversely correlated ($p < 0.005$) with their migration and EC50. Based on migration, proliferation and response to carboplatin PCA separated into four distinct groups. Using miRNA approach, we successfully identified miR-21-5p, 3p and miR-891-5p that were enriched in resistant cells and their exosomes. Transfected CAOV-3 cells (sensitive cells) with miRNAs showed a reduction in cells sensitivity to carboplatin. Finally, we were able to confirm the expression of these miRNAs in plasma from ovarian cancer patients.

Summary/Conclusion: We suggest that exosomal cargo may be used as prognostic biomarkers to monitor the response to treatments in patients with ovarian cancer.

PS10.03

Functional analysis of exosomes in cancer metastasis

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Introduction: The development of metastasis is a cause of death in many human cancers. Mechanisms for the acquisition of metastatic potential remain unknown. Recently, it has been reported that exosomes are a trigger of cancer metastasis. Exosomes are small vesicles that are secreted from cells and have been found to mediate signal transduction between neighbouring or distant cells. They have the tendency to specifically interact with target cells. In the future, it may be possible that exosomes can be used as biomarkers to predict the metastatic destination.

Methods: Established mouse Lewis lung cancer cells (low or high metastatic sublines) were examined about proliferation, migration, invasion and gaglioside expression by MTT assay, trans-well assay and flow-cytometry. Cells were inoculated into the mice subcutaneously or via tail vein, then tumour and metastatic tissues were observed by H&E stain. Cells from tumour sites were cultured then examined about proliferation and invasion ability. Exosomes were isolated from cell culture medium by differential centrifugation, and used for Western blotting. Cells treated by exosomes were analysed for malignant properties as described above.

Results: In proliferation, migration, and invasion assay, low metastatic subline showed lower proliferation, migration, invasion activity than high metastatic sublines. In flow-cytometry, high metastatic sublines showed decreased GM1 and GD1a expression levels compared with low metastatic subline. To examine metastatic ability, the cells were inoculated into mice. After 2–4 weeks, invasive- and metastatic- foci to distant tissues such as thigh muscle and lung were observed. To examine effects of exosomes on culture cells, cells were treated with isolated exosomes. As a result, low metastatic subline treated with high metastatic cell-derived exosomes showed increased proliferation, migration, and invasion activity, and increased phosphorylation of intracellular signalling molecules such as paxillin and Erk1/2. In turn, high metastatic subline treated with low metastatic cell-derived exosomes showed reduced proliferation, migration, and invasion activity, and phosphorylation of intracellular signalling molecules.

Summary/Conclusion: High metastatic subline-derived exosomes enhanced malignant properties in low metastatic sublines.

PS10.04

Profiling of circulating exosomal content across epithelial ovarian cancer and the role of exosomes in tumour progression

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Introduction: A significant proportion of patients with epithelial ovarian cancer (EOC) often present with advanced stage disease, when treatment options are limited. Therefore, it is essential to gain a better understanding of the tumour microenvironment to identify potential therapeutic targets. We profiled the exosomal content (miRNAs and proteins) of patients with EOC

and examined the effect of these exosomes on cells within the tumour microenvironment.

Methods: A cohort of 127 patients were included in this study. Exosomes were isolated and characterized from plasma obtained at different stages of EOC. A small RNA library was prepared, and the expression of specific miRNAs was validated using RT-qPCR. The protein profile was determined using Mass Spectrometry (MS/MS) and SWATH Analysis. Exosomal proteins and miRNAs were subjected to linear mixed modelling analysis using the lme4 package in "R". Fibroblast cells were incubated with patient-derived exosomes and monitored using the IncuCyte (TM), a live-cell imaging system. Cell proliferation and migration was determined over the course of the experiment and RNA and proteins were extracted after 48 hours. The expression of nine specific miRNAs was confirmed using RT-qPCR and the protein profile determined using MS/MS.

Results: Exosomal miRNAs and proteins demonstrated differential expression with advancing cancer progression, following at least three distinct patterns of change: increasing, constant or decreasing rate. Ingenuity Pathway Analysis analysis revealed that the exosomal content was associated mainly with cell–cell communication and cell migration. Functional analysis showed that exosomes increase fibroblast migration and proliferation in association with EOC progression (i.e. Stages I to IV). MS/MS identified 115 proteins differentially expressed between early stage and advanced stage-exosome treated cells. A comparison between control cells (no treatment) and treated cells showed a difference in the expression of 126 proteins, with tumour suppressor, Paired Box 1 and lysosomal trafficking protein, VPS41 expression, significantly lower in the treated cells ($p < 0.05$).

Summary/Conclusion: We propose that exosomes present in the circulation of EOC patients transfer oncogenic cargo to cells present in the tumour microenvironment to promote cancer progression.

PS10.05

Extracellular vesicle-mediated transmission of bone morphogenic proteins in Acute Myelogenous Leukaemia

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Introduction: Acute Myelogenous Leukaemia (AML) is an aggressive cancer originating from abnormal white blood cells of the bone marrow (BM). AML modifies the BM into a pro-leukaemic niche in part through the release of extracellular vesicles (EVs). We previously demonstrated that AML EVs decrease mature blood cell production, and traffic to stromal cells to induce osteogenesis. We hypothesized that AML cells utilize EVs to transmit regulatory factors to recipient BM cells to change the cellular composition of the BM and support cancer progression. Our studies confirmed that AML EVs contain bone morphogenic protein (BMPs) – historically though to be secreted growth factors – involved in formation of bone and maintenance of stem cells.

Methods: To identify the association of BMPs with AML EVs, we used both *in vitro* and *in vivo* xenograft models, and a combination of ELISA, flow cytometry, and super resolution microscopy.

Results: AML cells explanted from the BM display marked ER-stress in comparison to *in vitro* cultured cell types as an adaptive response to the tumour microenvironment. In AML blasts, the expression of BMP-2,4,6,7 mRNA strongly correlated with the activation of the unfolded protein response pathway (which acts to mitigate ER-stress). Inducing ER-stress in AML cells *in vitro* resulted in both an increase in BMP protein as well as total EVs produced. EVs released from these cells contained ~3-fold more BMP-2,6 over non-stressed cells, whereas the level of free-BMP-2,6 in supernatant remained unchanged. Exposing these purified EVs to BM stromal cells induced osteogenic differentiation and apoptosis. Additionally, in ER-stressed AML cells, BMP-2 localizes into CD63+ intracytoplasmic vesicles – indicative of pre-exosomal multivesicular bodies – further confirming the EV-BMP association. Thus far, AML cells have been found to release EVs that contain BMP-2 and −6, while additional BMP types remain to be tested.

Summary/Conclusion: Since we have shown that AML EVs rapidly accumulate within stromal cells in vitro and *in vivo*, altering cellular phenotypes, we propose that EVs concentrate and target functional BMPs directly to these cells to create a pro-leukaemic environment. EV trafficking of BMPs is a novel signalling mechanism that may explain phenotypic changes seen in leukaemic BM, and their role in leukaemic progression merits further study.

PS10.06

Exosomes derived from differentially invasive ovarian cancer cells modulate tumour growth and metastasis in vivo

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Introduction: Exosomes have been likened to a "fingerprint" of their originating cells, as they contain several bioactive molecules which can be delivered to the target cells at either local or distant locations. The delivery of these biomolecules can cause changes in gene expression and signalling in the target cells. Hence, in this study, we aimed to explore the impact of exosomes on tumour growth/ metastasis in a xenograft model and assess the changes in the proteomic content of tumour cells and exosomes from mice.
Methods: Exosomes were isolated from highly invasive SKOV-3 (exo-SKOV-3) and less invasive OVCAR-3 (exo-OVCAR-3) ovarian cancer cell lines. Exosomes (10 ug/mL) were injected into a xenograft model (n = 8/ group), twice a week for 6 weeks and the tumour growth was monitored using *In Vivo* Bioluminescence Imaging (IVIS). Tissue and circulating exosomes obtained from the mice were subjected to a quantitative mass spectrometry approach SWATH MS/MS, followed by ingenuity pathway analysis (IPA). Finally, we compared between the protein expressions profiles from the circulating exosomes and metastatic nodes or tumour growth.
Results: IVIS imaging indicated that the tumour burden in mice injected with exo-OVCAR-3 was higher than in mice injected with exo-SKOV-3 (p = 0.004). However, mice injected with exo-SKOV-3 had more tumour nodules throughout the peritoneal cavity. Proteomic analysis of the cancer tissue obtained from mice injected with exo-SKOV-3 compared to exo-OVCAR-3 identified the differential expression ($p < 0.05$) of 105 proteins. Interestingly, the protein profile in tumour tissue obtained from mice injected with exo-SKOV-3 was associated with the Wnt canonical pathway (β-catenin). Moreover, we found 36 proteins with differential expression in exosomes from mice treated with exo-SKOV-3, ($p < 0.04$). Finally, we identified 29 exosomal proteins that are highly associated with cancer metastasis and 21 proteins are associated with tumour growth.
Summary/Conclusion: These observations suggest that exosomal signalling plays an important role in ovarian cancer metastasis.

PS10.07

Cancer-associated fibroblast accelerate cancer metastasis through exosomes

Kim Kimin[a], Yeon Ju Hun[b], Sohn Yeh Joo[a], Ruri Lee[a], Yoo Hye Ju[a]

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Introduction: Exosomes are known to be important mediators between the primary and secondary sites for tumour progression and metastasis with their microenvironment. Exosomes released by cancer cells induce the cancer-associated fibroblasts, which create a niche to development cancer progression, making it more permissive cancer metastasis.
Methods: We have developed 3D tumour microenvironment model mimicking the interactions between cells and ECM by injecting of collagen gel for ECM to, and then, the formation of monolayer of cells for blood vessel. The exosomes were isolated from three different malignant cancer cells (i.e. from A431, B16BL6 and MDAMB231), and delivered into the channel in microfluidic device, then created a unidirectional flow by the difference in pressure gradient. We profile mRNAs of normal cell, CAFs with and without cancer cells in genetic analysis.
Results: We confirmed that various cancer-derived exosomes differentiated CAFs, facilitating metastasis in recapitulating the 3D tumour microenvironment in real time. The three difference CAFs have commonly enriched genes related to extracellular region for cellular response, and fibrinolysis to degrade ECM for biological process in genetic analysis. The migrated cancer cells followed by CAFs showed different specific molecular mechanisms, suggesting that the melanoma cells had MAPK related signalling, the squamous cancer cells had cell adhesion related signalling, and the breast cancer cells had inflammation, cytokine related signalling, which may contribute to the invasive progression of cancer.
Summary/Conclusion: The cancer-derived exosomes play an important role in modulating the tumour microenvironment, and induce CAFs to promote metastasis. The 3D microfluidic model showed the relationship between the CAFs and cancer cells invasion in real time in physiological manner and specific mechanism in a genetic manner.
Funding: This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the ministry of Education, Science and Technology (NRF-2016R1C1B2013345) and Samsung Research Funding Center of Samsung Electronics under Project Number SRFC-IT1701-00

PS10.09

The miR-27b in breast cancer exosomes

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Introduction: miR-27b has been shown to possess anti-tumour growth and anti-drug resistance activities in associated with breast cancer progression. Loss of miR-27b existed in the cancer cells can lead to the promotion of cancer cells. However, the precise mechanism of miR-27b loss is unclear, in particular, involving in tumour microenvironments and metastasis.

Methods: Here, we attempted to elucidate tumour-derived exosomes bearing miR-27b in regulating tumour microenvironments via modulation of cancer stem cell growth and migration.

Results: The expression level of miR-27b was decreased in tumour-derived exosomes in coincidence with progression of breast cancer, suggesting its negative role in tumour progression via modulating tumour microenvironments. Consistently, miR-27b showed a diminished trend in malignant breast cancer cell lines compared with the control cell line. To further examine the impact of miR27b in tumour microenviroments, we found that the formation of tumour associated fibroblasts (TAFs) and tumour associated macrophages (TAMs) were impacted by miR-27+ exosomes. Moreover, the increases in tumour migration and invasion were observed by miR-27b+ exosomes treated fibroblasts.

Summary/Conclusion: Therefore, we illustrated a simple mechanism of miR-27b attending in the progression of breast cancer. In the future, the manipulating the existence of miR-27b may be a novel strategy for breast cancer therapeutic.

PS10.10=OWP1.01

Mir-1227 alters extracellular vesicle shedding

Andrew R. Chin[a], Minyung Kim[b], Valentina R. Minciacchi[c], Tatyana Vagner[b], Javier Mariscal[b], Cristiana Spinelli[a], Mandana Zandian[b], Paolo Gandellini[d], Nadia Zaffaroni[d], Shivani Sharma[e], Sungyong You[b] and Dolores Di Vizio[a]

[a]Cedars Sinai Medical Center, West Hollywood, USA; [b]Cedars Sinai Medical Center, Los Angeles, USA; [c]Cedars Sinai Medical Center, Frankfurt, Germany; [d]Fondazione IRCCS Istituto Nazionale Tumori, Milan, USA; [e]University of California, Los Angeles, Los Angeles, USA

Introduction: Extracellular vesicles (EV) play a key role in cancer development and metastasis by influencing the behaviour of the primary tumour and by aiding the establishment of a pre-metastatic niche in distant organs. This process is due to the EV-mediated functional transfer of biologically active molecules including microRNA (miRNA). miR-1227 is a poorly characterized miRNA that is enriched in EV secreted by prostate cancer (PC) cells in comparison to non-tumourigenic prostate epithelial cells. However, the role of miR-1227 in cancer is poorly understood. Our objective is to determine the role of miR-1227 in PC.

Methods: RNA sequencing from miR-1227 stably expressing PC cells, RISCTRAP Immunoprecipitation of miR-1227 bound mRNA, and five different in silico miRNA target prediction methods were used to identify putative miR-1227 targets. Exosomes and large oncosomes (LO) were isolated by differential ultracentrifugation followed by density gradient purification. Atomic force microscopy and TRPS were used to quantify exosomes and LO secreted by PC cells stably expressing miR-1227 or vector control.

Results: A comparative analysis between different EV subtypes indicates that miR-1227 is enriched in LO, a class of EV that are secreted by highly invasive and metastatic amoeboid-migrating cells. LO carry more RNA than the more widely studied exosomes indicating that LO may be a more robust source of EV-encapsulated miRNA. Gene ontology analysis from miR-1227 targets identified by RNA sequencing from miR-1227 stably expressing PC cells, RISCTRAP Immunoprecipitation of miR-1227 bound mRNA, and in silico miRNA target prediction highlighted several genes related to EV secretion. miR-1227 alters the localization of exosome and LO markers in multiple cancer cell lines, and induces the shedding of LO while inhibiting the shedding of exosomes. Furthermore, miR-1227 induces the migration of poorly migratory cancer cells and increases the expression of tumour supportive cytokines.

Summary/conclusion: Together these data hint that miR-1227 may promote prostate cancer progression through several mechanisms including alteration of EV shedding.

Funding: 2017–2022 R01CA218526 Chesapeake urology associates Sanford J. Siegel, MD prostate cancer research scholarship Luke wu-jei chang discovery fund PI dod PCRP award PC150836

PS11: Stem Cells
Chairs: Kyoko Hida; Noriko Watanbe
Location: Level 3, Hall A

15:00–16:00

PS11.02

Bacterial endotoxin-preconditioned periodontal ligament stem cells induce M1 polarization of macrophage through extracellular vesicles

Hyejong Kang[a], Myung-Ju Lee[b], Sang Jung Park[b] and Myung-Shin Lee[b]

[a]Dankook University Sejong Dental Hospital, Sejong, Republic of Korea; [b]Eulji University School of Medicine, Daejeon, Republic of Korea

Introduction: Periodontitis is a common disease that characterized by chronic inflammation and tissue destruction of gums. To resist pathogenic microbes, gingival epithelial cells and inflammatory cells produce various pro-inflammatory cytokines, chemokines and enzymes. Human periodontal ligament stem cells (PDLSCs) derived from mature periodontal ligaments have stem cell properties similar to mesenchymal stem cells. PDLSCs possess not only differentiation potential to other tissues but also immunomodulatory abilities. Therefore, PDLSCs might be a vital role in the modulation of immune response. In this study, we investigated the effect of PDLSCs on the polarization of macrophages.
Methods: The polarization of macrophage cell line, THP-1 cells, was investigated on the conditioned media or extracellular vesicles (EVs) from PDLSCs that were pretreated with or without lipopolysaccharide. EVs were isolated from the conditioned media of PDLSCs by differential centrifugation and characterized. The functions of EVs on macrophage polarization and underlying mechanisms were analysed by RT-qPCR and ELISA,
Results: While the conditioned media from PDLSCs in normal culture condition did not affect the polarization of macrophage, lipopolysaccharide (LPS)-preconditioned PDLSCs induce significant changes in M1 polarization of macrophages. Extracellular vesicles (EVs) isolated from the conditioned media of LPS- preconditioned PDLSCs by centrifugal filter device (MWCO 100 kD) or differential centrifugation methods showed strong M1 polarization effect of macrophages. Additionally, M1 polarization was abolished by DNase I treatment on EVs.
Summary/Conclusion: Our results demonstrated that LPS-stimulated PDLSCs induce M1 polarization of macrophage through EVs, suggesting EVs from PDLSCs might be a potential therapeutic target for the inflammation in the periodontium.
Funding: This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF-2017R1A2B4002405).

PS11.03

Hypoxia enhances the angiogenic properties of adipose stem cell-derived extracellular vesicles in culture

Jolene Phelps, David Hart, Alim Mitha, Neil Duncan and Arindom Sen

University of Calgary, Calgary, Canada

Introduction: The widely recognized benefits of adipose stem cells (ASCs) in regenerative medicine have at least in part been attributed to the extracellular vesicles (EVs) that they secrete, which are known to deliver bioactive cargo to target cells. EVs can be isolated from spent medium following ASC population expansion in culture. It has been shown that manipulating the culture environment may impact the biological characteristics of EVs. Here we examined if the angiogenic properties of ASC-derived EVs are impacted by culture oxygen level, and tested their effect on cerebral microvascular endothelial cells (CMECs).
Methods: Ethically obtained human ASCs were cultured for 3 days in PPRF-msc6 serum-free medium under 3% (hypoxic) or 21% (normoxic) headspace O2 conditions. EVs were isolated from media via ultracentrifugation and evaluated for concentration (nanoparticle tracking analysis), and angiogenic factor content (Luminex technology). Functional assays (proliferation, tube formation) were carried out by culturing human CMECs in endothelial basal medium (EBM-2) supplemented with 2 different concentrations of ASC derived EVs. CMEC proliferation in tissue culture flasks was quantified using a Cyquant Proliferation Kit. Tube formation on Matrigel coated plates was quantified using ImageJ software. RT-qPCR was used to measure angiogenic gene expression levels in ASCs and CMECs for each test condition. All studies and analyses were carried out in at least triplicate.
Results: Hypoxia upregulated VEGF expression in ASCs 4.47 ± 0.24 fold ($p < 0.0015$) compared to normoxia and induced higher EV secretion. EVs obtained from hypoxic ASC cultures contained higher

concentrations of angiogenic proteins VEGF, HGF, PLGF and follistatin; and reduced concentrations of bFGF, endoglin, IL-6 and IL-8. The presence of ASC-derived EVs enhanced angiogenesis of CMEC cultures in a dose dependent manner as measured via enhanced proliferation, tube formation and upregulation of ANG-1, ET-1, TGF-β and VEGF expression.

Summary/Conclusion: The angiogenic properties of ASC-derived EVs may be enhanced through hypoxic culture. These EVs are able to promote angiogenesis of CMECs *in vitro* and may have utility in the treatment of ischemic injury.

Funding: Natural Sciences and Engineering Research Council of Canada

PS11.06

Production and use of extracellular vesicles-depleted human platelet lysate to improve large, clinical grade-compatible production of therapeutic human cell-derived extracellular vesicles

Philippe Mauduit[a], Sylvie Goulinet[b], Juliette Peltzer[c], Bastien Rival[c], Sebastien Banzet[c], Jean-jacques Lataillade[c] and Georges Uzan[b]

[a]Inserm, Villejuif, France; [b]INSERM, villejuif, France; [c]CTSA, CLAMART, France; [d]INSERM, Villejuif, France

Introduction: Human cells use multiple and sophisticated modes of communication. These include direct cellular communication, secretion of cytokines, chemokines or growth factors and also production of extracellular vesicles (EV) containing proteins, DNA, mRNA, miRNA. On the other hand, cell therapy using Mesenchymal Stromal Cells (MSCs) is getting a growing interest in a wide range of indications in human. In many cases, a substantial part of the therapeutic effects relies on cell-secreted factors and the extracellular vesicles (EV) are proposed as a cell-free surrogate for MSCs therapy. However, culture media commonly used for culturing cells requires serum or platelet lysate that contains large amounts of EV that cannot be distinguished and separated from the cell-secreted EV. Purification and characterization of EV therefore needs the prior elimination of contaminant EV contained in serum or Human Platelet Lysate (HPL). Serum-free media to produce EV may not be fully satisfactory since they often limit cell survival. Since regulatory authorities recommend avoiding animal components and xenobiotic-free culture conditions have to be considered for EV production. HPL offers such a possibility as it is useful substitute to FBS to isolate, amplify and maintain human cells.

Therefore, we describe a new procedure for GMP-compatible production of human cells-derived EV.

Methods: First, a Human Plasma Lysate (HPL) is produced from which the EV are removed by tangential-flow-filtration resulting in an EV-FREE HPL (EV depletion > 99%). Second, cells (grown in HPL-supplemented medium) are rinsed and placed in medium added with EV-FREE HPL. After 72 h, the medium is collected for EV quantification and replaced by fresh EV-FREE HPL supplemented media for a new production cycle.

Results: This method allows multiple production cycles and improved cell survival, cellular morphology and EV production. Following 3 × 72 h consecutive production phase, MSCs amplification would produce 2.4 and 2.7 more EV when incubated in the presence of, respectively, 5% and 8% EV-free HPL compared to HPL-free medium.

Summary/Conclusion: This process, compatible with the production of large volumes of conditioned media including in bioreactors, will allow large-scale production of therapeutic EV.

PS11.07

Synchronized cell differentiation via exosomes

Tomohiro Minakawa; Kae Nakamura and Jun K. Yamashita

[a]Laboratory of Stem Cell Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan

Introduction: Embryonic development proceeds in a highly orchestrated manner. It is assumed that synchronization of a timing of differentiation and cell fate among neighbouring cells is necessary for proper tissue development. However, the mechanism of synchronization is still largely unknown.

Methods: A mouse embryonic stem cell (ESC) line PKA-ESC, which can inducibly express constitutively active protein kinase A (CA-PKA), rapidly differentiates into mesoderm with PKA activation (depletion of doxycycline (Dox-)). We established a cell-chimeric culture system using two mouse ESC lines, PKA-ESC and Control-ESC to artificially generate a gap of timing in differentiation. We cocultured Control ESCs with PKA-ESCs to observe how they synchronously differentiate by overcoming the gap of timing in differentiation. Exosomes were collected from PKA-ESCs and added to Control-ESCs or mouse embryos. miRNA sequencing was performed comparing contents in exosomes from PKA-ESCs under Dox+ condition: control or Dox- condition: PKA activation, accelerated differentiation. We also established several ESC lines that encode miRNAs and performed coculture experiments with control-ESCs.

Results: After Dox-inducible activation of PKA, PKA-ESCs differentiate faster than Control-ESCs. In the coculture system, the timing of mesoderm differentiation of Control-ESCs were synchronized with faster differentiating PKA-ESCs (synchronized cell differentiation). Furthermore, addition of exosomes purified from PKA-ESCs promoted the differentiation of Control-ESCs. The exosomes also promoted mesoderm differentiation in postimplantation-stage mouse embryos. We found several miRNAs as the functional molecules in exosomes, and confirmed that miRNAs overexpressing cells can promote the differentiation of Control-ESCs in the coculture system.
Summary/Conclusion: We unveiled a novel cellular synchrony phenomenon and its mechanisms regulated by exosome-mediated cell communication, which would be broadly involved in tissue development.
Funding: This work was supported by JST CREST Grant Number [JPMJCR17H5 Japan].

PS11.08

Effects of mesenchymal stromal cells licensing on profile of extracellular vesicles

Giuliana Minani Bertolino[a], Tik Shing Cheung[b], Chiara Giacomini[c], Martin Bornhauser[d] and Francesco Dazzi[e]

[a]King's College London, London, United Kingdom; [b]King's College London, London, United Kingdom; [c]King's College London, London, United Kingdom; [d]King's College London; Technische Universität Dresden, Dresden, Germany; [e]King's College London, London, United Kingdom

Abstract: The roles of mesenchymal stromal cells (MSC) in the immune system are subject of increasing interest and of widening clinical applications. Recent evidences has shown that extracellular vesicles (EV) secreted by MSC can share some of the functional roles of their parental cells, among them the immunosuppression ability. Prior to exert immunomodulation, MSC effects rely on the presence of inflammatory mediators in the microenvironment: (1) proinflammatory cytokines such as IFN-γ and TNF-α, and (2) by the action of inflammatory effector cells which culminates on MSC apoptosis without the loss of immunomodulatory property. Therefore, we propose that different licensing of MSC can generate EV with distinct profiles and aspects on the immunomodulation.
Methods: To test this hypothesis, we characterized EV population derived from untreated MSC, MSC licensed by pro-inflammatory cytokines (IFNγ and TNFα) and from MSC undergoing apoptosis (anti-Fas antibody). We also isolated and characterized EV from plasma of Graft-versus-Host Disease (GvHD) patients receiving MSC as therapy (0, 4, 24, 48 h after MSC injection). EV size, shape and concentration were accessed by NTA and electron microscopy. MSC and EV surface markers were identified by bead-based flow cytometry. To study the EV contend, the presence of a panel of regulatory molecules was verified by qPCR and Western blot.
Results: We found that both MSC treatment generate population of EV heterogeneous in size, with main range between 100 and 200 nm and bigger vesicles (>500 nm) present in apoptotic MSC-EV samples. Apoptosis induction significantly increased the particle release. MSC-derived EV share mRNA and protein with their parental cells, and the different environment where the MSC is cultivated interfere in the EV content. Moreover, our preliminary data shown that GvHD patients receiving MSC have increased EV containing MSC-related suppressive molecules straight after cell infusion.
Summary/conclusion: In summary, our results show that the different environment where MSC is cultivated interfere on their EV content, and can provide a signature of the "licensed" MSC. This was further tested in patients undergoing MSC treatment with a view of identifying biomarkers for pharmacokinetics studies.
Funding: This work was supported by the Bloodwise Specialist Programme and by CAPES – Brazil.

PS11.09

Effects of mesenchymal stromal cells licensing on profile of extracellular vesicles

Giuliana Minani Bertolino[a], Tik Shing Cheung[a], Chiara Giacomini[a], Martin Bornhauser[b] and Francesco Dazzi[a]

[a]King's College London, London, United Kingdom; [b]King's College London; Technische Universität Dresden, Dresden, Germany

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LBS01: Late Breaking- EV Therapeutics
Chairs: Xabier Osteikoetxea; Akiko Takahashi
Location: Level 3, Hall A

15:00–16:00

LBS01.01

Mesenchymal stromal cells derived-extracellular vesicles effect on microglia cells

Dorota Kaniowska[a], Kerstin Wenk[b], Franziska Lange[a], Sebastian Greiser[a], Ulf-Dietrich Braumann[a] and Yarua Jaimes[c]

[a]Fraunhofer IZI, Leipzig, Germany; [b]Institute for Clinical Immunology, University of Leipzig, Leipzig, Germany; [c]ISEV, Leipzig, Germany

Introduction: Mesenchymal stromal cells (MSCs) are a heterogeneous population of cells with very high self-renewal properties and the capacity to induce tissue regeneration and reduce inflammation. Extracellular vesicles (EVs) from MSCs have shown to have immune modulatory properties and given their small size, are good candidates as therapeutic agents for tissues of difficult access, such as the central nervous system (CNS). Microglia cells are the CNS immune cells and are involved in the progression of the degeneration in many neuroinflammatory diseases. We evaluated the interaction of MSC-EVs with microglia cells and their effect as regulators of activation.
Methods: We have used an *in vitro* model for stimulation of the BV-2 microglia cell line and primary cells with lipopolysaccharides (LPS) and amyloid β aggregates. Real time PCR methods were used to assessed the transcripts upregulation of tumour necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6, nitric oxide synthases (iNOS), Prostaglandinendoperoxide synthase 2 (PTGS2) and chemokine ligand (CCL)-22. Protein levels of TNF-α, IL-1β and IL-6 were evaluated by ELISA and cytometric bead arrays. Live cell imaging approaches were used to evaluate the interaction of MSC-EVs with microglia cells *in vitro*.
Results: We demonstrated that MSC-EVs are actively internalized by microglia cells. Moreover, that presence of MSC-EVs prevents transcription and protein expression of pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, inducible isoform of nitric oxide synthases (iNOS) and prostaglandinendo peroxide synthase 2 (PTGS2) upregulation by microglia cells towards LPS and amyloid β. Furthermore, MSC-EVs suppressed the phosphorylation of the extracellular signal kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK) and the p38 MAP kinase (p38) molecules given in response to LPS stimulation.
Summary/conclusion: MSC-EVs are strong modulators of microglia activation. The modulatory activity of MSC-EVs can be of major impact in the treatment of neuroinflammatory diseases.
Funding: This project is co-financed with tax money from the state of Saxony, Germany. High Performance Center of Chemical and Biosystem Technology: Grant 100312141, Grant 100321061. YJ is financed by a TALENTA Financing award from the Fraunhofer Society.

LBS01.02

Porcine milk exosomes protect intestine against deoxynivalenol damage

Mei-Ying Xie[a], Ting Chen[a] and Yong-Liang Zhang[b]

[a]South China Agricultural University, Guangzhou, USA; [b]college of animal science, south china agricultural university, Guangzhou, China (People's Republic)

Introduction: Deoxynivalenol (DON) serious damage intestinal vulnerable structures and intestinal integrity. Our previous study showed that exosomes could facilitate intestinal cell proliferation and neonate intestinal tract development, but the protection of milk exosomes of damage caused by DON is unclear.
Methods: Neonatal Kunming mice were given 0.4 ml porcine milk exosomes or saline for 3 weeks and then given 2.5 mg/kg bw/day DON for 7 days. Intestinal morphology was assessed using H&E. Cells viability are tested by MTT, Edu and cell counting assay. WB, qRT-PCR and immunofluorescence were used to show the effects of porcine milk exosomes on the damages of intestine and IPEC-J2 cells caused by DON. At last, bioinformatics Analysis, luciferase reporter assay was to verify the potential targeting relationship between miRNAs and mRNAs.
Results: Porcine milk exosomes significantly alleviated the negative effects of DON on body weight and the damage degree of intestinal epithelial. In addition, these exosomes significantly reversed the inhibition of DON on cell proliferation and intercellular tight junction-associated proteins, such as levels of β-catenin, p-Akt, cyclinD1 and claudin1, and decreased the

apoptosis-related protein p53 and p21. *In vitro*, porcine milk exosomes significantly attenuated the damage of DON on cell viability, proliferation and tight junctions, consistent with the results *in vivo*. Our results also indicated that porcine milk exosomes up-regulate the expression of miR-181a, miR-30c, miR-365-5p and miR-769-3p in cells and downregulated their targeting genes in p53 pathway, such as FAS, TP53, SERPINE1.

Summary/conclusion: Porcine milk exosomes protected intestine and IPEC-J2 cells against DON damage, and encapsulated miRNAs play a role in regulating p53 pathway. Our study opened a new sight in breast milk exosomes, which may contribute to intestinal health during the neonatal period

Funding: This work was supported by grants from the National Natural Science Foundation of China [grant numbers 31472163], and The Chinese National Key Scientific Project (2016YFD0500503).

LBS01.03

Exosomal PD-L1 embedded with thermoresponsive gel promotes wound healing

Dandan Su[a], Zhanxue Xu[b], Hongbo Chen[b], Fang Cheng[b] and Xiangyi Cai[c]

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Introduction: Wound healing is a complex process involving multiple cell types with different roles, which is divided into stages of haemostasis, inflammation, proliferation and remodelling. As many chronic wounds are the result of excessive and chronic inflammation, we hypothesized that effective wound repair could be achieved by inhibiting overactive immune cells to the injured skin. The PD-1/PD-L1 immune checkpoint pathway prevents excessive tissue destruction during inflammatory states, and PD-L1 expression is induced by pro-inflammatory factors in multiple cell types throughout the body. Interestingly, recently PD-L1 is found to exists in extracellular vesicles (EVs) as a transmembrane protein. Thus we would like to test if exosomal PD-L1 would regulate the immunity and inflammatory response to promote proper wound healing.

Methods: Exosomal PD-L1 were isolated from melanoma cells stimulated with IFN-γ by differential centrifugation and were characterized by flow cytometry, TEM, DLS, zeta potential, Western blot and confocal microscopy. Exosomal PD-L1 were administered in a mouse skin injury model through a thermoresponsive hydrogel, which was gelatinized at body temperature to preserve exosomal PD-L1 throughout the wound. Flow cytometry analysis, qPCR, HE staining and immunohistochemical analysis were performed to further explore the therapeutic effect of exosomal PD-L1 at the tissue levels.

Results: Exosomal PD-L1 in thermoresponsive gel led to a decreased T cell activation, indicated by CD4, CD8, and IL-2 markers. In the presence of exosomal PD-L1, there was also an increased expression of growth factors, which significantly promoted wound contraction and wound re-epithelialization.

Summary/conclusion: Collectively, our current findings suggest that exosomal PD-L1 speeds up wound healing when applying into a novel thermoresponsive gel on top of the injured skin, which provides a new perspective for using immunotherapy to promote tissue repair and regeneration.

Funding: F. Cheng would like to thank Sigrid Jusélius foundation, the National Natural Science Foundation of China (Grant no. 81702750) and the Basic Research Project of Shenzhen (Grant no. JCY20170818164756460) for funding.

LBS01.05

Intranasal delivery of mesenchymal stem cell derived exosomes loaded with PTEN siRNA repairs complete spinal cord injury

Shawei Gou[a], Nisim Perets[b], Oshra Betzer[c], Shahar Ben-Shaul[d], Anton Sheinin[e], Izhak MichaelevskiMichaelevski[f], Rachela Popovtzer[c], Daniel Offen[g] and Shulamit Levenberg[h]

[a]Department of Biomedical Engineering, Technion-Israel Institute of Technology, Israel; [b]Sagol School of neuroscience, Tel Aviv University, Israel, Tel aviv, Israel; [c]Faculty of Engineering and the Institute of Nanotechnology & Advanced Materials, Bar-Ilan University, Israel, Ramat Gan, USA; [d]Department of Biomedical Engineering, Technion-Israel Institute of Technology, Haifa, 3200002, Israel, Haifa, Israel; [e]Sagol School of Neuroscience, Tel Aviv University, Israel., Haifa, Israel; [f]Department of Molecular Biology, Ariel University, Israel.; [g]Sagol School of neuroscience, Tel Aviv University, Israel, Sacklar school of medicine, department of human genetics and biochemistry Tel Aviv University, Israel., Tel Aviv, USA; [h]Department of Biomedical Engineering, Technion-Israel Institute of Technology, Israel, Haifa, Israel

Introduction: Complete spinal cord injury (SCI) is a debilitating disease which usually leads to permanent functional impairments, with various complications and limited spontaneous recovery or efficient treatment. Here, we report that in rats with complete SCI, intranasal administrations of mesenchymal stem cells-derived exosomes (MSC-Exo) could penetrate the blood brain barrier, home selectively to the spinal cord lesion, and show affinity to neurons within the lesion. When these exosomes were loaded with phosphatase and tensin homolog small interfering RNA, termed ExoPTEN, they migrated from the nose and silenced PTEN expression in the lesion. Furthermore,

the loaded exosomes promoted robust axonal regeneration and angiogenesis, accompanied with decreased astrogliosis and microgliosis. Moreover, the intranasal ExoPTEN treatment partially restored electrophysiological and structural integrity, and most importantly, enabled remarkable functional recovery. This rapid, non-invasive approach, using cell-free nano-swimmers carrying molecules to target pathophysiological mechanisms, suggests novel strategy for clinical translation to SCI and beyond.

Methods: MSC-exo were extracted from Human bone marrow mesenchymal stem cells. All rats had complete transection of the spinal cord. MSC-exo were loaded with co-incubation together with siRNA for PTEN conjugated to cholesterol. The MSC-exo were given by intranasal administration 1–4 h post SCI.

Results: Here we show that SCI rats that were intranasally treated with MSC-exo present functional improvement in motor and sensory output. The MSC-exo were homed in the SCI area and led to reduction in inflammatory markers, increased angiogenesis and regrowth of transected axons. MRI and electrophysiological measurements were done to show the axonal recovery and signal transduction

Summary/conclusion: Exosomes derived from Human bone marrow mesenchymal stem cells and loaded with inhibitor molecule for PTEN pathway were found efficient in ameliorating complete transection of the spinal cord via intranasal administration, including remarkable functional improvement.

LBS01.06

ASC-EXOSOME as a potential therapeutic for atopic dermatitis

Byong Seung Cho[a], Jin Ock Kim[b], Dae Hyun Ha[a] and Yong Weon Yi[a]

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Introduction: Atopic dermatitis (AD) is an inflammatory disease that has rapidly increased in the prevalence in recent decades. Despite the high demand for AD therapy, current treatment options are limited and have potentially harmful side effects. Recently, several clinical studies highlighted human mesenchymal stem cells (MSCs) as novel potential therapeutics for suppressing allergic progress in the AD, and the majority of their therapeutic effects is mediated their secretome which contains exosomes. There are, however, several drawbacks for the therapeutic use of MSCs, such as poor engraftment efficiency, non-specific differentiation, and short half-life, etc. Otherwise, exosomes can be off-the-shelf since they are not live, expecting to overcome the limitations of MSC easily and become powerful alternative therapeutics. Here, we investigated the therapeutic effects of exosome from adipose tissue-derived MSC (ASC-EXOSOME) on atopic dermatitis in two *in vivo* models.

Methods: ASC originated from adipose tissue of a healthy donor. ASC-EXOSOME was isolated from ASC conditioned media through a sequential filtration method. AD-like skin lesions were induced in mice by applying house dust mite antigen or a chemical irritant. After administration of ASC-EXOSOME either subcutaneously or intravenously the anti-inflammatory effects were demonstrated by measuring serum IgE level, immunostaining of immune cells, real-time PCR, etc.

Results: Systemic administration of ASC-EXOSOME dose-dependently lowered serum IgE level and the number of eosinophils in AD mice blood, and reduced mast cell infiltration and up-regulated mRNA levels of IL-4, IL-31, IL-23 and TNF-α in the skin lesions compared to AD control. Skin barrier function was also improved by ASC-EXOSOME.

Summary/conclusion: Systemic administration of ASC-EXOSOME dose-dependently lowered serum IgE level and the number of eosinophils in AD mice blood, and reduced mast cell infiltration and up-regulated mRNA levels of IL-4, IL-31, IL-23, and TNF-α in the skin lesions compared to AD control. Skin barrier function was also improved by ASC-EXOSOME.

LBS01.07

Porcine milk exosome miRNAs attenuate lipopolysaccharide-induced apoptosis by inhibiting TLR4/NF-κB and P53 pathways

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Introduction: Intestinal epithelial cells are important for pathogen infection. LPS is an endotoxin and induces intestine inflammation. Milk exosomes improve the intestine development and immune system of newborn. The objective of this study is to investigate the protective mechanisms of porcine milk exosomes in rescuing LPS-induced intestinal epithelium injuries.

Methods: Both *in vivo* and *in vitro* tests were carried out to confirm protection of porcine milk exosome on LPS induced injury to intestine.

Results: *In vivo*, exosomes protected the jejunum integrity and health from LPS damage through H&E results and attenuated LPS-induced pro-inflammatory factors

secretion through ELISA results. *In vitro*, we got similar results in the intestinal epithelial cell line IPEC-J2. Bioinformatics analyses and cell experiments results shown exosome miR-4334, miR-219 reduced pro-inflammatory responses and miR-338 inhibited LPS-induced apoptosis of intestinal epithelial cells via TLR4/MyD88/NF-κB and P53 pathway, respectively. Co-transfection of those three miRNAs had the best effect on resisting LPS-induced IPEC-J2 apoptosis than any one of these three miRNAs.

Summary/conclusion: In conclusion, porcine milk exosomes protected the intestine against LPS-induced injury through decreasing cell inflammatory and resisting cell apoptosis by exosome miRNAs. This study expands our understanding of bioactive molecules in milk and provides new strategies for developing functional foods in the future.

Funding: This work was supported by grants from the National Natural Science Foundation of China [grant numbers 31472163], and The Chinese National Key Scientific Project (2016YFD0500503).

LBS01.08

Extracellular vesicles from mesenchymal stromal cells for the treatment of radiological burns

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Introduction: High-dose acute radiation accidents of industrial and medical origin and the risk of a terrorist act (NRBC) have been taken into consideration for some years. The work carried out by our teams led to a new therapeutic approach for the management of victims of accidental irradiation, consisting of autologous Mesenchymal Stromal Cells (MSCs) injection associated with reparative surgery. Preclinical studies showed that MSCs, mainly by their secretory activity, contribute to control inflammation, promote angiogenesis and tissue regeneration. MSC-derived extracellular vesicles (MSC-EVs) might be key mediators of MSC function. This project aims to propose an innovative therapy product based on the use of Extracellular Vesicles (EVs) for the treatment of radiological burns following accidental irradiation.

Methods: MSCs were grown until reaching 80% confluence, then moved to EV collection medium for 72 h. EVs were purified by tangential flow filtration followed by size exclusion chromatography and characterized by Nanoparticle tracking analysis. MSC-EVs were evaluated *in vitro* in an inflammatory assay using human monocytic THP-1 cells treated with lipopolysaccharide, with or without co-culture with MSCs or EVs. The level of pro-inflammatory TNFα in the culture supernatant was measured by ELISA assay. EVs were also evaluated *in vivo* using a mouse model of acute hind limb radiation injury. Cell therapy products (1x106 MSCs or a range of 2.45E+10, 4.90E+10 or 9.80E+10 MSC-EVs/animal) were intramuscularly injected 14 days post-irradiation. Macroscopic analysis of injury was performed at regular intervals.

Results: Preliminary results showed an immunomodulatory effect of MSCs-EVs, as shown by their ability to reduce TNFα secretion by THP-1 cells in response to LPS. Moreover, *in vivo* results showed a decrease of injury score in animals injected with the highest EV concentration at day 10 and 14 post-injection.

Summary/conclusion: These preliminary results suggest a beneficial effect of MSC-EVs on the healing process of cutaneous radiation syndrome and could represent a valuable therapeutic alternative in the context of radiological emergency. Further exploration of the molecular mechanisms is now necessary.

Funding: French Direction Générale de l'Armement, under contract ANR-16-ASTR-0024

LBS01.09

Adipose-derived stem cells enhance chondrogenesis and cartilaginous matrix synthesis of articular chondrocytes is mediated by extracellular vesicles

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Introduction: To date, mesenchymal stem cells including adipose-derived stem cells (ADSCs) have been intensively investigated as a cell-based therapy to treat articular cartilage damages in both animal and human studies. However, the detailed mechanism of how ADSCs regenerate the damaged articular cartilage remains unclear. Increasingly, studies present evidence that ADSCs mediate tissue repair via secretion of trophic factors on damaged tissue. In this study, we test the hypothesis that ADSCs-derived extracellular vesicles (EVs) enhances chondrogenesis and matrix synthesis of human articular chondrocytes.

Methods: Human ADSCs were labelled with CM-DiI and then pre-cultured in DMEM supplemented with 2% FBS for 48 h to induce EVs release. After induce

EVs release, the conditioned medium derived from pre-cultured with ADSCs were isolated, and then was used to treat articular chondrocytes. There were three groups in the study: (1) Control: articular chondrocytes treated with DMEM supplemented with 2% FBS without pre-cultured with ADSCs, (2) Conditioned medium: articular chondrocytes treated with DMEM supplemented with 2% FBS, which is pre-cultured with ADSCs, (3) Conditioned medium remove EVs: articular chondrocytes treated with conditioned medium, which the EVs were removed by ultracentrifugation. At the indicated time point, the chondrocytes were harvested for further analysis including cell proliferation, chondrogenic gene expressions (Collagen type II), and cartilaginous matrix synthesis (Glycosaminoglycan synthesis).
Results: Intercellular communication occurs through EVs. EVs transferred into chondrocytes can be found in the conditioned medium group. However, there is no EVs transfer in the conditioned medium removed EVs. There is no significant difference in cell proliferation of chondrocytes among three groups. The chondrogenic gene expression and cartilaginous matrix synthesis of chondrocyte is significantly enhanced in conditioned medium group when compared with control group. Moreover, there is no significant difference between control and conditioned medium removed EV groups.
Summary/conclusion: ADSCs enhances chondrogenesis and matrix synthesis of human articular chondrocytes is mediated by EVs

LBS01.10

Application of milk exosome for leaping cosmeceutical materials.

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Introduction: Milk is one of the best exosome materials widely used as an ingredient in various foods. Although the antibacterial effect present in milk has been long known, however studies related to the antibacterial activity associated with milk exosomes are fairly limited. The purpose of this study is to suggest the possibility of using the antimicrobial effect of milk exosomes in cosmeceutical field.
Methods: Commercially available non-fat milk-based on Pasteur treatment was used. Milk was centrifuged at 210,000 *g* for 70 min at 4□. TEM and cryo-EM was used to determine the shape of milk exosomes and its size was measured using qNano (iZon, Australia). For antimicrobial activity test, *Staphylococcus aureus* (*S. aureus*) was cultured in LB broth medium at 37□, O/N. The seed culture ratio (1/100, 1/1000) and different exosome concentration were inoculated and growth was confirmed by time.
Results: The average size of the MiExo obtained was 120 ~ 140 nm. Both TEM and cryo-EM image showed a typical exosome shape morphology. The Western blotting confirmed the detection of TSG101 marker, which is a representative marker of MiExo. The antimicrobial activity of S. aureus was determined at different conditions. It exhibited 2.5 times antimicrobial effect when the MiExo and the bacteria were inoculated together at an early stage in log phage (10^8 CFU/mL). Based on the inoculation dilution factor(DF), very high antimicrobial effect of approximately 19 times was observed for 1/1000 DF as compared to the 1/100 DF. S. aureus hardly grew in the experiment group with 1/1000 DF. The antimicrobial efficacy based on the amount of exosome was 13 times higher for 10^11 particles as compared to 10^6 particles.
Summary/conclusion: The extraction of MiExo and its antimicrobial effect was determined. The antimicrobial effect of MiExo performed in this study is considered to be stable with low side effects and has great potential as a superior natural material in the future cosmeceutical market.
Funding: This work was carried out with the support of “Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ012653)” Rural Development Administration Republic of Korea.

LBS01.11

Control of neural stem cell differentiation to generate defined exosome populations

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Introduction: Exosomes derived from the clinical grade neural stem cell line *CTX* (ReNeuron) are the basis of a new class of therapy for the treatment of degenerative disorders. Since exosomes contain a subset of molecules derived from their parent cell, progenitor and differentiated *CTX* may generate exosomes with diverse phenotypes. It is vital that these are well characterized to allow robust manufacture and

isolation of particular exosome populations and to understand their implications in therapeutic applications

Methods: Screening of support matrices (microcarriers) and substrates for growing CTX was performed in a bespoke microfluidic device for 7 days. Cells were then fixed and stained before applying automated imaging and analysis to determine the differentiated state of the cells. The process was repeated with a reduced panel of matrix/substrate combinations to study differentiation and exosome agonists for a period of 6 weeks as a means to accelerate CTX differentiation and increase exosome production. The conditions selected for each cell type were validated in a model bioreactor system at the 0.1L scale and the resultant exosomes characterized in terms of particle number, size distribution, miRNA content and CD markers

Results: The microfluidic screening approach allows the study of a panel of 336 matrix, substrate, differentiation agonist and exosome agonist/antagonist combinations enabling the experimental space to be reduced by >98% prior to any scale-up activities, thereby minimising experimental time, cost and risk of failure. Our validation successfully achieved our target cell population of 60,000 cells/cm2 in 4 days and found that the resultant exosomes had miRNA and CD marker profiles dependent on stage of differentiation of the culture

Summary/conclusion: CTX were successfully adapted for growth on microcarriers in a suspension bioreactor system to provide a scalable platform for progenitor and differentiated CTX-derived exosome production. The exosome characteristics change in terms of both CD markers and miRNA profile according to the differentiated state of their parent cell. This has implications on not only their therapeutic function and potency but also the design of processes for their manufacture and purification in order to deliver consistent product profile

Funding: Innovate UK

LBS01.12

Engineered stem cell membrane-cloaked gold nanorods for efficient cancer therapy

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Introduction: Faced with various limitations in how functionalization of nanoparticles‘ surfaces with synthetic organic materials, researchers have begun to envision strategies for using bioinspired materials as an alternative. In particular, nanoparticles require many improvements *in vivo* to increase stability, enhance circulation time and distribution and improve dissolution. For this reason, directly transferring the cell membrane to the surface of the nanoparticles, the complexity of the lipid, protein and carbohydrate-containing cell membranes can be faithfully maintained, allowing the coated nanoparticles to have characteristics that appear to the source cell. The behaviour of cells *in vivo* is usually determined by cell membrane proteins. This method of directly coating the cell membrane also has the same characteristics of nanoparticles depending on the characteristics of the source cells.

Methods: First, in order to coat the cell membrane, the capped CTAB for the surface stabilization of the gold nanorod was replaced with a negatively charged citrate. And, extracted stem cell membranes are engineered using lipid-tethered peptide for enhancing targeting specificity to cancer cell. Engineered cell membrane was cloaked onto the surface of gold nanorods using simple bath sonication. It can be confirmed that the stem cell membrane is well-coated on the surface of gold nanorod through various analysis including, zeta potential, TEM, PAGE gel and Western blotting, etc.

Results: Citrate-capped gold nanorods are characterized using zeta potential and TEM. And, zeta potential of well-established cell membrane-cloaked gold nanorods is similar to that of cell membrane. Cell membrane coating onto gold nanorods is also visualized using TEM with negative staining. After cell membrane coating, the protein marker in the cell membrane is detected identically to the cell membrane, but the nuclear marker is not detected in Western blotting analysis.

Summary/conclusion: Cell membrane coating is a platform technology that provides an easy top-down method for designing nanocarriers with surfaces that replicate very complex and diverse functions needed for effective bio-interfacing and give nanoparticles more functional.

Funding: This study was supported by the National Research Foundation of Korea (NRF) Grants funded by MEST (NRF-2017R1A6A3A040123620)

LBS02: Late Breaking- EVs in Intercellular and Interorganism Communication
Chairs: Hanne Winther-Larsen; Kallen Sullivan
Location: Level 3, Hall A

15:00–16:00

LBS02.01=OWP1.14

Annexin V binding modulates the response of macrophages to mesenchymal stromal cell-derived extracellular vesicles

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Introduction: We have previously shown that Annexin a5 (An5) binding to mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) enhances the anti-inflammatory properties of these nanoparticles in an animal model of colitis. However, the mechanisms underlying these effects are unknown. Here, we investigated the immunoregulatory effect of MSC-EVs with and without An5 binding on activated macrophages *in vitro*.

Methods: Macrophages were isolated from mouse bone marrow and activated by INFgamma and LPS. Clinical grade Wharton Jelly-derived MSC-EVs were obtained from The Cell Factory (Esperite NV, Niel, Belgium) and quantified by Resistive Pulse Sensing analysis. 5,0E+05 macrophages were incubated with PBS (vehicle only, control, group 1) 5,0E+08 MSC-EVs (group 2), 5,0E+08 MSC-EVs added with 2 ug An5 (group 3) or with 2 ug free An5 (group 4). After 24 h, the cells were analysed by flow cytometry and RNA was extracted for RT-PCR analysis.

Results: Incubation with MSC-EVs significantly increased only the expression of IL-10 in IFNgamma/LPS-activated macrophages. Incubation with An5-MSC-EVs resulted in a significant induction in the expression of both pro- and anti-inflammatory cytokines, including TNFalfa, IL-1Beta, IL-6, IL-10 and TGFbeta1. Incubation with free An5 induced only pro-inflammatory cytokines without affecting IL-10 and TGFbeta1 expression. The iNOS2/Arg1 ratio was reduced in both EV-treated groups, indicating a shift from M1 to M2 polarization.

Summary/conclusion: In conclusion, both MSC-EVs and An5-MSC-EVs shift the macrophage phenotype from M1 to M2. The combined induction of TGFbeta1 and IL-10, observed only in An5-MSC-EV-stimulated macrophages, might be related to the immune-modulating characteristics of these modified EVs that contribute to the therapeutic effects observed *in vivo*.

Funding: The BROAD MEDICAL RESEARCH PROGRAM AT CCFA supported this work

LBS02.02

PD-L1/CTLA-4 nanovesicles have an immunosuppressive effect on a mouse skin graft model

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Introduction: Skin transplantation has been employed to serious injuries, but a potent inflammatory immune response often leads to rejection of allogeneic skin grafts. T-cell activation by immune allorecognition is a major cause to trigger acute rejection. Immune checkpoint pathways such as the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)/Cluster of differentiation 80 (CD80) provide an immunosuppressive environment, preventing excessive tissue destruction due to inflammatory immune response. Thus we would like to see if bioengineering cell membrane derived nanovesicles (NVs) to display PD-L1 and CTLA-4 would reduce immunological rejection through enhancing PD-1/PD-L1 and CTLA-4/CD80 immune inhibitory axis.

Methods: We established HEK 293T cells that stably express PD-L1/CTLA-4 on the cell membranes and prepared cell membrane nanovesicles. Confocal microscopy and immunoprecipitation analysis were used to determine the interaction of PD-1/PD-L1 and CTLA-4/CD80 on the cell membrane. After that, T-cell activation and proliferation were examined by flow cytometry analysis of a panel of markers. Finally, we tested the

effect of PD-L1/CTLA-4 NVs on relieving skin grafting *in vivo*.

Results: We successfully engineered cell membrane derived nanovesicles to display PD-L1/CTLA-4, which were characterized by transmission electron microscopy and Western blotting In addition, confocal microscopy showed that PD-L1/CTLA-4 NVs can interact with PD-1 of and CD80 of target cells. Furthermore, PD-L1/CTLA-4 NVs led to reduction of T cells activation and proliferation. Finally, compared to control mice, the skin-grafting mice had a high response rate to relieve immunologic rejection when treated with PD-L1/CTLA-4 NVs.

Summary/conclusion: As a summary, NVs containing dual molecular targets PD-L1/CTLA-4 exhibit strong immune inhibitory effect, promoting the healing of grafting skin. Thus, PD-L1/CTLA-4 dual immune blockade by nanovesicles provides a promising strategy to inhibit skin graft rejection.

LBS02.03

Exosome-mediated horizontal gene transfer: a possible driving force behind mammalian genome evolution & a new risk for genome editing

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Introduction: The CRISPR-Cas9 system has been successfully applied in many organisms as a powerful genome editing tool. We have previously reported that unintentional DNA sequences derived from retrotransposons, genomic DNA, mRNA and vectors are captured at double-strand breaks (DSBs) sites when DSBs are introduced by the CRISPR-Cas9 system. Therefore, it is possible that unintentional insertions associated with DSB repair represent a potential risk for human genome editing gene therapies. To address this possibility, comprehensive sequencing of DSB sites was performed, and we found that bovine DNA fragments were captured at DSB sites in fertilized mouse eggs and cell lines.

Methods: We determined the lengths of the indels introduced by the CRISPR-Cas9 system *in vivo* and *in vitro* by deep sequencing of PCR products amplified with two primers across the target DSB site.

All animal studies were conducted in accordance with the guidelines approved by the animal care committee of the National Institute of Health Sciences.

Results: To determine the origin of bovine DNA fragments, we used goat serum, rabbit serum, and exosome-free FBS instead of FBS in the cell culture medium. Goat BovB and rabbit LINE1 sequences were horizontally transferred to DSB sites, however, almost no bovine DNA sequences were captured, suggesting that these horizontal gene transfers were mediated by exosomes.

Summary/conclusion: We demonstrated that horizontal gene transfer assisted by CRISPR-Cas9 occurs in NIH-3T3 cells and mouse embryos. This phenomenon might be the driving force behind mammalian genome evolution. In fact, mice with fusions between the murine Peg10 gene and a bovine SINE were obtained. A number of possible trans-species horizontal gene transfer events have been reported in mammals. Exosomes are present in all fluids from living animals, including seawater and breathing mammals, suggesting that exosome-mediated horizontal gene transfer is the driving force behind mammalian genome evolution. The findings of this study also highlight an emerging new risk for this leading-edge technology.

Funding: AMED (18mk0104073j0103 &18ak0101093j001), Health Sciences Research Grants from the Ministry of Health, Labor, and Welfare, Japan (H30-KAGAKU-IPPAN-002 & H30-KAGAKU-SHITEI-001), and JSPS (26430183 and 18K19315)

LBS02.04

Comparative studies on *in vitro* and *in vivo* inflammatory activities of extracellular vesicles and soluble factors derived from bacteria

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Introduction: Soluble factors released by cells play important roles in intercellular communication. However, extracellular vesicles (EVs) have recently attracted much attention as intercellular communicasomes, complex extracellular organelles that mediate intercellular communication. While it has been reported that EV-associated molecules elicit greater activities than soluble forms, no studies have compared the activities of EVs as a whole with soluble factors. In this study, EVs and soluble factors derived from bacteria were compared with regard to local and systemic inflammatory activities.

Methods: *Escherichia coli* was cultured in a chemically defined medium, and conditioned medium (CM) was harvested from the culture. EVs and soluble factors

(CM-EVs) were isolated from 3 kDa cut-off concentrated CM by ultracentrifugation. RAW264.7 cells were treated with EVs and CM-EVs, then the release of TNF-α and IL-6 were measured with ELISA. In addition, wild-type mice were intraperitoneally administered with EVs and CM-EVs, and septic signs were observed. Inflammatory indices including the concentrations of TNF-α and IL-6 as well as the numbers of infiltrated immune cells were also assessed from the peritoneal lavage fluid, serum and bronchoalveolar lavage fluid.
Results: EVs mediated the release of IL-6 from RAW264.7 cells *in vitro*, with greater extent than CM-EVs. Unlike CM-EVs, EVs mediated systemic septic symptoms including hypothermia, eye exudate formation and leukopenia. While both EVs and CM-EVs mediated immune cell infiltration into the peritoneum, EVs mediated the elevation of the concentrations of TNF-α and IL-6 in the peritoneal lavage fluid, more efficiently than CM-EVs. In addition, EVs mediated the elevation of the concentrations of TNF-α and IL-6 in the serum, whereas, CM-EVs did not. More importantly, EVs mediated immune cell infiltration as well as the elevation of the concentrations of TNF-α and IL-6 in the bronchoalveolar lavage fluid, whereas CM-EVs did not.
Summary/conclusion: Although EVs and soluble factors mediated local inflammatory responses, only EVs can act as long-range effectors in systemic inflammatory responses, suggesting EVs as new therapeutic targets for infectious diseases.

LBS02.05

Gram-negative bacterial extracellular vesicles promote angiogenesis by inducing interleukin-6

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Introduction: Angiogenesis, the formation of blood vessels from pre-existing vasculature, is an essential complex process for multiple pathophysiological conditions including bacterial infection, inflammatory diseases and bacterial sepsis. Several pathological functions of Gram-negative bacterial extracellular vesicles (EVs), also known as outer membrane vesicles have been shown to induce local inflammation, systemic inflammation, and septic shock, and so on. However, no studies have assessed the effects of Gram-negative bacterial EVs on angiogenesis.
Methods: *Escherichia coli* EVs were subcutaneously administered to wild-type mice, along with Matrigels. The Matrigels were subjected to whole mount immunostaining, and vascular area was measured. As macrophages are involved in angiogenesis, macrophage infiltration was also assessed in the Matrigels. Peritoneal macrophages from wild-type mice were treated with *E. coli* EVs, and the conditioned media were treated to endothelial cells to measure cell migration. Furthermore, to show the role of interleukin-6 (IL-6) on angiogenesis, *E. coli* EVs were subcutaneously administered to wild-type and IL-6 knock-out mice, along with Matrigels. Then, the Matrigels were subjected to whole mount immunostaining, and vascular area was measured. In addition, peritoneal macrophages from wild-type and IL-6 knock-out mice were treated with *E. coli* EVs, and the conditioned media from the macrophages were treated to endothelial cells to measure cell migration.
Results: *E. coli* EVs promoted *in vivo* angiogenesis and macrophage infiltration in wild-type mice. Peritoneal macrophages from wild-type mice, treated with *E. coli* EVs, mediated endothelial cell migration *in vitro*. However, *E. coli* EVs did not promote angiogenesis and macrophage infiltration in IL-6 knock-out mice. Furthermore, peritoneal macrophages from IL-6 knock-out mice, treated with *E. coli* EVs, did not mediate endothelial cell migration.
Summary/conclusion: Gram-negative bacterial EVs have potent angiogenic activities by promoting macrophage infiltration and inducing IL-6. These findings provide insights into the effects of Gram-negative bacterial EVs on bacterial infection-related pathological diseases including bacterial infection, inflammatory diseases, and bacterial sepsis.

LBS02.06

Dendritic cell derived-exosomes activate immune systems by transferring exosome involved factors to T cell

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Introduction: Exosomes released from dendritic cells (DCs) are responsible for the persistence of antigen presentation. So, we considered that whether DCs-derived exosomes could induce suppress cancer cells and more effective response of an immune system and

what factors in exosomes-involved DCs can activate T cells.

Methods: Luciferase gene transferred-3LL cells (murine lung cancer cell line derived C57BL/6) were injected into C57BL/6J mice by intraperitoneal administration. And then, DCs, DCs-exosomes or 3LL-exosomes were weekly administrated to lung cancer-bearing mice. The exosomes derived from DCs decreased lung cancer cell growth compared with DCs, DCs-exosomes and non-treated. We evaluated mRNA expressions differences in between LPS-treatment DCs and none treatment DCs with DNA microarray analysis.

Results: DNA microarray analysis data showed that 44 genes increased as ratio LPS-treated DC mRNA vs non-treatment DC one. Western blot analysis showed one of the genes contained higher in exosomes derived from LPS-treatment DCs than that derived from non-treatments.

Summary/conclusion: This gene induces T cell proliferation and signals for T cell maturation. We concluded that DCs derived-exosomes activate anticancer immune systems by transferring exosome-involved the factor to T cells.

LBS02.07

Crosstalk between endoplasmic reticulum stress and autophagy in kidney diseases

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Introduction: The endoplasmic reticulum (ER) regulates several cellular functions, including the protein biosynthesis, folding, trafficking and modification. The accumulation of unfolded or misfolded proteins causes a form of cellular stress that has been termed ER stress. ER stress activates the unfolded protein response (UPR) signalling network which serves as an adaptive response. The potential benefit of maintaining ER homeostasis modulates ER stress status to protect the kidney against various pathogenic environments. Furthermore, ER stress induces autophagy in mammalian cells. The ER stress-induced autophagy offers protection from oxidative-induced cytotoxicity and ameliorated kidney injury. In this study, we understand the mechanism modulated the regulation of UPR and autophagy in kidney cells.

Methods: We examined cytotoxicity of ER stress inducers (tunicamycin (TM) or thapsigargin (TG)) in human kidney cells HK-2. To analyse low doses TM or TG induces autophagy using immunofluorescence microscopy, transmission electron microscopy and Western blot analysis. Also, to investigate TM or TG inhibits oxidative stress through the induction of autophagy. In addition, we established an adenine-induced chronic kidney disease (CKD) mice model. The effectiveness of TM or TG was investigated in CKD mice model.

Results: Low concentrations of TM and TG did not affect cell viability in HK-2 cells. TM and TG induced UPR pathway and autophagy. Furthermore, TM and TG inhibited oxidative stress-induced cell death. The inhibition of autophagy can increase cytotoxicity in HK-2 cells. Therefore, TM- and TG-induced autophagy palys a protective role. The inflammasome and cytokine synthesis were suppressed after treatment with TM and TG. In addition, HK-2 stimulated with TM or TG had increased production of exosomes. In the model of adenine diet-induced CKD, TM and TG ameliorated renal dysfunction and injury through the induction of ER stress and autophagy.

Summary/conclusion: These results suggest that TM and TG protected kidney cells against oxidative stress-induced cell death and inhibited inflammatory effect. ER stress-induced autophagy may be a pro-survival role. However, the complete mechanism of how TM and TG regulate exosomes is yet unknown and need further investigation.

LBS02.08

Extracellular Vesicle-induced protein phosphorylation: Rapid activation of epithelial-mesenchymal transition pathways in lung epithelial cell

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Introduction: Extracellular vesicles are important mediators of cell-to-cell communication. With their bioactive cargos including proteins, lipids and nucleic acids, they can alter the fate of a recipient cell. Mast-cells and lung epithelium exists in close physical proximity and activity in mast cells is reflected in epithelial cells. In this study, we hypothesized that mast cell-

derived EVs alter recipient epithelial cells by inducing phosphorylation of multiple proteins.

Methods: Mast cells derived-EVs (HMC1.1) were obtained by differential ultracentrifugation. We determined the early protein phosphorylation induced by EVs, in recipient cell A549 cells using phospho-protein microarray (Sciomics), and determined the longer-term effects on RNA transcripts and protein changes in epithelial cells.

Results: Prolonged exposure of EVs altered cellular morphology of recipient epithelial A549 cells. This was in line with changes in the transcript that are known to activate epithelial-mesenchymal transition (EMT), including increased levels of TWIST1, MMP9, TGFB1, and BMP-7. This was also reflected at the protein levels in recipient cells; e.g downregulation of CDH1 and upregulation of MMP. By contrast, EMT inducing transcription factor Slug-Snail was upregulated. To determine any rapid responses 30 minutes after EV treatment we performed phospho-protein microarray of recipient cells. In-silico analysis of phospho-proteome revealed proteins in signalling networks that are part of the PI3K-Akt pathway or cytokine receptor interactions. Interestingly, a protein involved in regulating focal adhesion and tight junctions was phosphorylated in these experiments; e.g. CLDN1, OCLN, and ACTN1. Finally, we validated one of the well-studied EMT-regulating pathway (TGFβ signalling) in both A549 and BEAS-2B cell lines.

Summary/conclusion: Mast cell-derived EV facilitates activation of EMT in lung epithelial cells, which is closely associated to EMT-associated protein phosphorylation. This study highlights the component of signalling pathways that are rapidly phosphorylated in recipient cells with the contact of EVs.

Funding: VBG group Herman Krefting Foundation, Swedish Cancer Foundation, Swedish Research Council, and Heart and Lung Foundation, EAACI, AG Foundation, Lundgren Foundation, Sahlgrenska University Hospital, and Sahlgrenska Academy.

LBS02.09

Serum extracellular vesicular miR-21-5p is a predictor of the prognosis in idiopathic pulmonary fibrosis

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Introduction: Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease for which no treatment is capable of providing a complete cure. The median survival for IPF patients from the time of diagnosis is approximately 3 years. IPF patients differ in terms of the disease progression rate and prognosis, complicating the prediction of survival. The identification of prognostic predictors for IPF is important for determining who requires the most intensive therapies. In this study, we explored the possibility that microRNAs of serum EVs changed during lung fibrosis and could serve as prognostic biomarkers of IPF.

Methods: To determine target microRNAs in IPF, we measured serum EV microRNA expression profiles using microRNA PCR arrays in a bleomycin mouse lung fibrosis model. Secondly, we enrolled 41 IPF patients and conducted a 60-month prospective cohort study. Expression of serum EV miR-21-5p was normalized by dividing by the EV amount. The relative amount of EVs was measured using the ExoScreen method. We calculated the correlations between baseline serum EV miR-21-5p expression and other clinical variables. Furthermore, we determined if serum EV miR-21-5p can predict mortality during 60 months using the Cox hazard model. According to the median level, we divided the IPF patients into two groups. Then we compared the survival rate during 60 months between the two groups using the Kaplan-Meier method.

Results: Serum EV miR-21-5p was elevated in both the acute inflammatory phase (day 7) and the chronic fibrotic phase (day 28) in the mouse model. In the clinical setting, serum EV miR-21-5p was significantly higher in IPF patients than in control subjects. The baseline serum EV miR-21-5p was correlated with the rate of decline in vital capacity over 6 months. Furthermore, serum EV miR-21-5p was independently associated with mortality during the following 60 months, even after adjustment for other variables. In the survival analysis, IPF patients whose baseline serum EV miR-21-5p was high had a significantly poorer prognosis over 60 months.

Summary/conclusion: Our results may suggest that serum EV miR-21-5p has potential as a prognostic biomarker for IPF.

Funding: This work was supported by Grants-in-Aid for Scientific Research (24591148 and 15K09206 to M Yamada) from the Japan Society for the Promotion of Science (JSPS).

LBS02.10

Extracellular vesicles subpopulations: who are the key players in vascular inflammation?

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Introduction: Current conceptual insights on the mechanistic aspect of cell signalling by discovering extracellular vesicles (EV) have shed light on the pathological understanding of chronic inflammatory diseases. The biological function of EV bulk in the progression of inflammation-associated disorders has become clear, the protein composition and function of the disease-specific subsets are often hampered by undesirable EV co-isolates. Thus, there is an urgent need to isolate certain subpopulations to generate a disease-relevant signature while retaining their functional integrity. Therefore, we aimed to fractionate the inflammation associated-EV subsets based on two important characteristics (sedimentation and surface markers) and subsequently profiling the immunomodulatory protein content.

Methods: TEM, NTA and Western blot were used to characterize the purified inflammation-associated EV subsets from TNF-α treated HUVEC based on their sedimentation speeds (10K and 110K) and surface markers (CDs and ICAM-1). Protein arrays were used to discover the immunomodulatory content of subsets. In addition, functional integrity of the EV subpopulations was assessed using migration cell based assays.

Results: We demonstrated that HUVEC upon inflammation release two distinct populations of heterogeneous EV, differing in size and quantity. The immunoaffinity of these two populations towards EV classical markers (a cocktail of CD9, CD63 and CD81) and an inflammatory-associated marker revealed that the circulating form of ICAM-1 is abundantly docked on the membrane of large EV, thus offering a potentially promising biomarker for immunocapturing of EV subsets. Moreover, protein profiling of EV size-based populations and their inflammation-associated EV subsets showed that the patterns of cytokines and adhesion markers were significantly different. In cell-based assays, EV of different sizes work synergistically in accelerating the vascular inflammation.

Summary/conclusion: A procedure of two purification steps resulted in purer inflammation-associated EV isolates, allowing a better understanding of their biology and functions at the onset of vascular inflammation.

Funding: This work was co-financed by the EU through the Interreg IV Flanders-the Netherlands project Interreg V Flanders-the Netherlands project Trans Tech Diagnostics (TTD).

LBS03: Late Breaking- EV Biogenesis, Loading, and Uptake
Chairs: Samarjit Das; Wang Jiang
Location: Level 3, Hall A

15:00–16:00

LBS03.01=OWP1.15

Membrane-radiolabelled exosomes for comparative biodistribution analysis in immunocompetent and immunodeficient mice – A novel and universal approach

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Introduction: Exosomes have gained interest as novel drug nanocarriers due to their biological origin and role in intercellular biomolecule delivery. In-depth knowledge of their *in vivo* biodistribution is therefore essential. This work aimed to develop a reliable and universal method to radiolabel exosomes to study *in vivo* biodistribution in mice.

Methods: Melanoma (B16F10 cells)-derived exosomes (ExoB16) were isolated and characterized for size, yield, purity, exosomal markers and morphology using Nanoparticle Tracking Analysis (NTA), protein measurements, flow cytometry and electron microscopy. Two radiolabelling approaches were explored – intraluminal labelling (111Indium entrapment via tropolone shuttling); and membrane labelling (111Indium chelation by covalently attached bifunctional chelator). Labelling efficiency and stability was assessed by gel filtration and thin layer chromatography. Melanoma-bearing immunocompetent (C57BL/6) and immunodeficient (NSG) mice were injected intravenously with radiolabelled ExoB16 (1x1011 particles) followed by metabolic cages study, whole body SPECT-CT imaging and ex vivo gamma counting at 1, 4 and 24 h post-injection.

Results: Membrane-labelled ExoB16 (ML-ExoB16) showed superior radiolabelling efficiency and radiochemical stability compared to intraluminal-labelled ExoB16 (IL-ExoB16). Both IL- and ML-ExoB16 showed prominent accumulation in liver and spleen. IL-ExoB16 showed higher tumour accumulation than ML- ExoB16 (6.7% and 0,6% ID/g tissue, respectively), with the former showing similar value as its free tracer ([111]Trop). The superior stability of the membrane-labelling approach rendered its result more reliable and was used to compare ExoB16 biodistribution in melanoma-bearing immunocompromized (NSG) mice. Similar biodistribution profile was observed in both C57BL/6 and NSG mice, where prominent accumulation was seen in liver and spleen, apart from the lower tumour accumulation observed in the NSG mice.

Summary/conclusion: Membrane radiolabelling of exosomes is a reliable approach that allows for both live imaging and quantitative biodistribution studies to be performed on potentially all exosome types without engineering parent cells.

LBS03.02

Rala and ralb finely tune EVs biogenesis and promote metastasis

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Introduction: Tumour extracellular vesicles (EVs) promote tumour progression. However, their behaviour in body fluids remains mysterious. In addition, further understanding of molecular mechanisms driving their biogenesis is needed to develop strategies aiming to impair their tumorigenic potential. We recently showed that the zebrafish embryo can be used to track and assess the function of circulating tumour EVs *in vivo* and provide a high-resolution description of their dissemination and uptake (Hyenne et al., Dev Cell, 19). We provided a first description of tumour EVs' hemodynamic behaviour and showed that they are rapidly taken up by endothelial cells and blood patrolling macrophages and subsequently stored in degradative compartments.

Methods: In addition, we recently investigated the molecular mechanisms of EV release in a tumorigenic context, using a mouse model of breast cancer carcinoma.

Results: We observed that depletion of either RalA or RalB GTPases decreases levels of EVs' secretion (Hyenne et al. JCB 15) and modifies their protein and RNA content. We further showed that RalA and B are required to properly localize PLD1 on MVBs thereby inducing EVs biogenesis. Interestingly, EVs secreted from RalA and RalB depleted cells are less prone to

endothelial permeabilization *in vitro*. Finally, RalA and RalB depletion significantly impairs lung metastasis in a syngeneic model of breast carcinoma suggesting that RalA/B controls lung metastatis by tuning the levels and contents of tEVs.

Summary/conclusion: Overall, our recent works proves the usefulness and prospects of zebrafish embryo to track tumour EVs and dissect their role in metastatic niches formation *in vivo*. It further provides new mechanistic information as to how RalA and RalB control the biogenesis of potent tumour-promoting EVs.

LBS03.03

In vivo visualization of extracellular vesicles released from mature osteoblasts by intravital multiphoton microscopy

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Introduction: Bone remodelling is essential for maintaining bone architecture and systemic mineral homeostasis throughout life. In the process, the formation of bone matrix by osteoblasts follows the removal of mineralized bone by osteoclasts. Despite intensive investigations on understanding their functions, the detailed mechanisms on their dynamic nature *in vivo* remain unknown. In this study we especially focus on the dynamics of mature osteoblasts which replenish bone matrices during homeostasis.

Methods: To understand the cellular dynamics of mature osteoblasts *in vivo*, here we established a reporter system where mature osteoblasts express enhanced cyan fluorescent protein (ECFP). We could visualize their dynamic nature *in vivo* by using intravital multiphoton microscopy for live bone tissues which we have originally developed so far.

Results: We detected that mature osteoblasts spontaneously release large extracellular vesicles (EVs), whose sizes are from 0.2 to 1 μm, and those are also taken up by mature osteoblasts. Such phenomenon could also be reconstituted in mature osteoblasts cultured ex vivo. Further analyses are currently ongoing in order to analyse the physiological and pathophysiological functions of these vesicles.

Summary/conclusion: This is the first study detecting the real dynamic nature of microvesicles *in vivo*, which are actively released from mature osteoblasts in the bone cavity. We think these microvesicles are important regulators for normal bone homeostasis as well as pathological remodelling.

LBS03.04

New products from donated blood – unravelling the potential of blood cell derived EVs

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Introduction: Extracellular vesicles (EV) originated from different cell types have recently been under intense investigation. Platelet EVs constitute the major fraction of EVs in the circulating plasma, however, there are only a few studies characterizing the populations of platelet concentrate derived EVs in more detail. Few recent publications show that plasma EVs can target specifically into certain mononuclear cell populations but little is known about their biological function, signalling and communication. As just recently addressed by Onodi et al., EV purification has major challenges as majority of EVs from plasma has lipoprotein particles and the abundant plasma proteins as impurities complicating the study of the role of pure EVs. We have previously shown that platelet concentrates used for transfusions contain increasing amount of EVs after longer storage period. It is important to study these platelet-EVs in more detail in order to understand their role in product functionality.

Methods: Excess platelet concentrates not needed for the clinical use were obtained from the Finnish Red Cross Blood Service. All donated blood products used for research were obtained from healthy volunteers who had given their informed consent. In our ongoing work we compared ultracentrifugation based isolation methods and size exclusion chromatography in order to collect differing populations of platelet concentrate derived EVs. EVs are further labelled with fluorescent surface protein, lipid and RNA markers and studied using Amnis ImageStream®X Mark II Imaging Flow Cytometer. Purity and characteristics of these isolated EVs are compared and their targeting into different mononuclear cells as well as their immunological relevance are investigated.

Results: Based on our results we are able to say that we get a pure population of EVs with low contamination of lipid or plasma protein impurities. The main population of the platelet concentrate derived EVs are platelet derived and thus CD41 positive, however, the origin of EVs differ as well as their cargo indicating differences in their immunological functions.

Summary/conclusion: Our aim is to find previously ignored, new applications for donated blood components and to identify the potential EV population to be

utilized either as therapeutic components in tissue repair or as drug delivery vehicles.

LBS03.05

The effect of rhinovirus type 16 derived microvesicles on the growth of hela cells

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Introduction: Belonging to group A, Rhinovirus Type 16 (HRV16) uses the receptor Intercellular Adhesion Molecule (ICAM) 1 to enter cells. Studies demonstrated Extracellular Vesicles (EV) discharge from viral infected cells harbour and distribute regulatory factors to recipient cells. These include viral RNA and proteins, viral and cellular miRNA, as well as host functional genetic elements to nearby cells, leading to the production of infections particles and modulating cellular responses including the spread or limitation of infection conditional on the type of pathogen and target cells. Here, we demostrate that HRV16 derived microvesicles (HRV16MV) infect HeLa cells at a higher rate than HRV16 particles.
Methods: HRV16MV were extracted from HeLa cells after 24 h of HRV16 infection (MOI 0.2) via ultracentrifugation. Quantified by Flow cytometry, HeLaMV control (HelaMVc) and HRV16MV were added to each well containing the HeLa cells treated with CGM (exosome free). The plates were incubated at 37°C in a 5% CO2 and left untreated for 24 h. HeLa cells control samples were observed against MV treated cells and the concentration/mL and % viability were determined every 4 h after 12 h incubation period to determine the effect of MV on the growth of the cell line.
Results: HRV16MV treated cells showed a growth decline after 16 h into the experiment, which suggests a faster infection rate (P**) when compared to HRV16 infection. Both, HRV16 and HRV16MV treated cells demonstrated a decline in % viability (P>**) after 16 h of infection in comparison to HeLaMVc. However, despite a slight decrease in in the growth rate of HRV16MV treated cells no statistical significance was observed in % viability between samples.
Summary/conclusion: HRV16MV treated cells showed an advanced infection rate of treated HeLa cells. HRV16 genome encodes two proteases specifically, 2A and 3C as well as a precursor protease, 3CD. These proteases are necessary for adequate virus replication, for the precise localisation of proteins during infection and for the temporal regulation of 2A and 3CD/3C protease activities during HRV16 infection. Therefore, these proteases may be hypothesized to be embedded in HRV16MV suggesting that they could potentially be hijacked by the virus to spread infection.

LBS03.06

A highly efficient cell-free protein synthesis system from plasmid DNA.

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Introduction: Protein is essential molecules that play many critical roles in the body. Activity and interaction of gene encoding proteins are recognized as a key element of pharmaceutical and biomaterials. However, since the general protein production method is performed through microorganisms and cell culture processes that require a lot of time and labour, it is not only decomposed and deformed by enzymes produced by cell growth but also has a limit to producing a large amount of protein. This study reports a DNA hydrogel that can induce excessive protein without lives cells using denatured DNA plasmid. This novel synthesis method improved the efficiency of protein expression through Rolling circle amplification that repetitive sequencing of nucleotides for protein expression in plasmid DNA that codes a specific gene. Notably, our synthesized DNA plasmid gel expressed large scale green fluorescence protein (GFP) than wild type. In addition, DNA gel has the strength of having a physical characteristic and being able to express the specific protein in a specific area. Novel large-scale protein expression method using cell-free DNA gel has great potential as a biomaterial for therapeutic protein delivery. In addition, this method can be applied mRNA manipulation, which plays a major role in transmitting proteins, can be used to transfer particles using RCA method and to produce vesicles containing proteins by putting them in vesicles.
Methods: The plasmid pT7CEF1-Chis was prepared Thermofisher. Linear single stranded circular DNA oligonucleotides from denatured DNA plasmid were incubated with T4 polymerase at 30� 72 h with dNTPs.
Results: We developed the periodically repeated nucleotides through RCA process band its cell-free largescale protein expression system. The synthesized plasmid DNA hydrogel has stable formation and having a high efficiency to express specific protein.
Summary/conclusion: In this study, we developed cell-free protein expression method, which were

synthesized by a uniquely designed RCA method, as a protein therapeutics platform technology. The cell-free protein producing DNA plasmid gel can be considered as a potential candidate for protein delivery.

LBS03.07

Targeting mevalonate pathway with low doses of valproic acid reduces large EVs shedding in different solid tumours

Chiara Ciardiello[a], Rossella Migliorino[a], Federica Iannelli[a], Maria S. Roca[a], Francesca Bruzzese[a], Biagio Pucci[a], Susan Costantini[a], Elena Di Gennaro[a], Alessandra Leone[a] and Alfredo Budillon[b]

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Introduction: Epithelial to mesenchymal transition (EMT) as well as mesenchymal to amoeboid transition (MAT) are linked with increased cancer cell motility and stemness, MAT being also described to favour large extracellular vesicles (EVs) shedding. Recently, both these phenotypic changes were associated to metabolic control involving the mevalonate pathway (MVP), a key controller of lipid metabolism but also a regulator of cell structure and signalling. valproic acid (VPA), an antiepileptic and a well-known histone deacetylase inhibitor, showed antitumor activity and capability to augment anticancer efficacies of other therapeutic approaches (i.e. ionizing radiation, chemotherapy, immunotherapy).
Methods: Two different isogenic models developed by our group were used: prostate cancer DU145 cells and their derived more aggressive subline DU145R80 selected as resistant to MVP-pathway inhibitors and enriched in stem markers; the colorectal cancer CO147 primary cell line, cultured either as differentiated cells or as cancer stem cells enriched spheres. Western blotting and metabolomics were performed to monitor MVP modulation upon VPA treatment (0.5-1 mM). Large EVs were isolated from cell media by discontinuous density gradient ultra-centrifugations and measured by Tunable resistive pulse sensing or flow cytometry VPA-treated or untreated cells.
Results: Both DU145R80 cells and CO147 cultured as spheres showed enriched stem like features and higher large EVs shedding, compared to parental DU145 and differentiated CO147 cells, respectively. At very low doses, VPA reduced large EVs shedding in both DU145R80 and CO147 sphere cultures, compared to the untreated cells, without affecting cells viability. Mechanistically, preliminary data suggest that VPA-induced effect is mediated by MVP pathway modulation.
Summary/conclusion: Our results describe for the first time a novel anticancer potential of VPA, being able to impact cancer cell to cell communication by reducing the shedding of large EVs.
Funding: AIRC FIRC

LBS03.08

The role of glycogen synthase kinase 3 beta in the biogenesis of extracellular vesicles by modulating microtubule dynamics

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Introduction: Extracellular vesicles (EVs) are spherical, bilayered membranous vesicles secreted by all living cells. EVs harbour various bioactive materials, and play diverse roles in biological processes such as tumour progression. There are several reports studied on the proteins involved in EV biogenesis mainly focused on the proteins involved in vesicle trafficking. However, proteins regulating EV biogenesis are still unclear. As most cellular processes are regulated by protein phosphorylation, which is regulated by kinases and phosphatases, identifying kinases and phosphatases involved in EV biogenesis helps to understand EV-mediated pathophysiological functions.
Methods: To identify kinases and phosphatases involved in EV biogenesis, a total of 76 kinase inhibitors and 33 phosphatase inhibitors were treated to A549 cells. The amounts of CD81, an EV-enriched protein, were quantified from the conditioned media to show alterations in EV biogenesis. To further verify the role of glycogen synthase kinase 3 beta (GSK3β) in EV biogenesis, stable cell lines expressing wild-type, constitutively active mutant, and dominant-negative mutant GSK3β were established, and alterations in EV biogenesis were measured in these cell lines. As microtubule dynamics affects EV biogenesis, changes in microtubule dynamics were also assessed in these cell lines.
Results: Among the kinase and phosphatase inhibitors, an inhibitor of GSK3β and calcineurin decreased and increased EV biogenesis, respectively. EV biogenesis was increased in the conditioned media from cells expressing constitutively active mutant GSK3β, and decreased in the conditioned media from cells expressing dominant-negative mutant GSK3β, when compared with cells expressing wild-type GSK3β. Microtubules were more disorganized in cells expressing constitutively active mutant GSK3β, and more aligned in cells expressing dominant-negative mutant

GSK3β, when compared with cells expressing wild-type GSK3β.

Summary/conclusion: By high-throughput screening of kinase/phosphatase inhibitors, we identified GSK3β as a positive regulator of EV biogenesis by modulating microtubule dynamics. These observations suggest that GSK3β as a novel therapeutic target against several diseases by modulating EV biogenesis.

LBS03.09

Post-translational modifications affects trafficking of hyaluronan synthase 2 and the release of extracellular vesicles

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Introduction: Hyaluronan synthase 2 (HAS2) is the major producer of Hyaluronan (HA) in adult vertebrates. Its enhanced expression has been lately related within the apical filopodia growth and the budding of extracellular vesicles (EVs). Moreover, a fraction of HAS enzymes are secreted from PM into extracellular vesicles (EVs), often covered by HA. We studied whether the mutations blocking post-translational modifications on HAS2 also affected the EVs released.

Methods: Site-directed mutagenesis was used to block ubiquitination (K190R), and phosphorylation (T110A) HA was measured using ELSA

Isolation of EV secreted by HAS2-transfected cells was performed using ultracentrifugation

Analysis of extracellular vesicles (EV) was performed with a Nanoparticle Tracking Analyzer and 3D culture

Results: Cell cultures transfected with HAS2 wt secreted ~50% more EVs as compared to mock controls. Similar stimulation of EV secretion was found with K190R, while non-increase of EVs occurred with T110A. These results lead us to two conclusions. First, PM residence of HAS2 is likely required for the stimulation of EV secretion. And second, HA synthesis is not strictly necessary for EV secretion, since K190R is enzymatically inactive. Cells were grown in a 3D matrix to check if K190R was entering itself in the vesicles. The data show that HAS2 wt and K190R, but not T110A were present in the EVs. This indicates that the mechanism of HAS2 stimulation of EVs involves HAS2 incorporation in them, and without the involvement of HA. Unexpectedly, 4-MU (HA synthesis inhibitor 4-MU) blocked the shedding of all transfected HAS2 and its mutants.

Summary/conclusion: Our data show that an enzymatically inactive HAS2 residing in PM (K190R) enhanced EV secretion to the same extent as HAS2 wt, while it did not induce the PM protrusions. Just the insertion of HAS2 in PM must, therefore, trigger a signal or structural alteration in the membrane that facilitates its inclusion in, and shedding of the EVs.

Another interesting finding was that while HA was not necessary for EV formation, the HA synthesis inhibitor 4-MU blocked HAS2 insertion in the EVs. This may represent yet another mechanism of HA synthesis inhibition by 4-MU. Exploring the mechanism of the block and its importance in HA synthesis and EV shedding will be interesting targets of future studies, especially in cancer epidemiology.

LBS03.10

Improving the stability of the large extracellular loop of human tetraspanin CD81

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Introduction: Members of tetraspanin protein family are abundant on the surface of nearly every type of extracellular vesicles (EVs) and are therefore attractive targets for modification, leading to transformation of the EVs into a targeted drug delivery system. The engineering of tetraspanin extracellular domains as independent folding units towards specific antigen recognition is therefore of particular interest.

Methods: We have applied rigid body protein modelling approach to design more stable mutants of large extracellular loop (LEL) of human tetraspanin protein CD81. Proteins were expressed in ExpiCHO expression system and IMAC-purified. Their stability was examined using DSC and the protein fold integrity assessed with HPLC-SEC in native conditions and reactivity with structurally dependent binding anti-CD81 antibody. Mutants based on such stabilized scaffolds were engrafted with human transferrin receptor (hTfr) specific peptide at different positions, tested for their biophysical properties and internalization *in vitro*.

Results: In order to enhance the tolerance for modification we successfully identified positions that could accommodate pairs of point mutations to cysteine residues, leading to de novo disulphide bridges in the human CD81 LEL. We achieved an increased thermal stability with a shift in melting temperature (Tm) of up to 25°C in mutants with one additional disulphide bridge. Mutants harbouring a combination of 2 engineered disulphide bonds showed an increased Tm of up to 43°C.
The graft of a hTFR-binding peptide into the D-Helix of the wild-type LEL resulted in a protein that still exhibited a compact fold. When the same peptide sequence was inserted between the helices A and B, the mutant showed an aberrant profile in SEC, which could be cured by using a scaffold variant with a stabilized LEL backbone. Additionally, both peptide-grafted proteins revealed increased internalization into hTFR-overexpressing SK-BR-3 breast cancer cells compared to the respective wild-type proteins.
Summary/conclusion: These results define important requirements for improving the amenability of tetraspanins, in particular CD81 LEL, for their engineering into a more versatile protein scaffold, which should empower the design of antigen-binding tetraspanins as targeting moieties of EVs and functionalize them as a drug delivery vehicle.

LBS03.11

Comparison of exosomes and ferritin protein nanocages for the delivery of membrane protein theraqeutics

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Introduction: Exosomes are small membrane vesicles secreted by most cell types that plays an important role in intercellular communication. Due to the characteristic of transferring their biomacromolecules, exosomes have potential as a new alternative for delivering protein therapeutics. Here, we investigate whether exosomes provide crucial advantages over other nanoparticles, in particular protein nanocage formulations, as a delivery system for membrane protein therapeutics. We characterized membrane-scaffold–based exosomes and protein-scaffold–based ferritin nanocages, both harbouring SIRPα (signal regulatory protein α), an antagonist of CD47 on tumour cells.
Methods: For preparing exo-SIRPα, HEK293T cells were transiently transfected with desirable plasmid DNA. Following a further incubation for 48 h, the supernatants of transfected HEK293T cells were harvested and subjected to a serial centrifugation protocol (300 × g for 10 min, 2000 × g for 10 min and 10,000 × *g* for 30 min) to remove debris. Then, exosomes were isolated from the cell culture medium by ultracentrifugation (150,000×*g* for 2 h). Ferritin-SIRPα and monomer SIRPα proteins were purified through an Ni-NTA chromatography step. For the impartial comparison, we adjusted the same amount of SIRPα proteins of 2 nanocages in all experiments.
Results: Exo-SIRPα exceeds Ferritin-SIRPα in all experiments, cell binding ability, enhancing phagocytic function of bone marrow derived macrophage, *in vivo* anti-tumour effect and tumour specific immune response. Exosome-SIRPα shows better feasibility compared to ferritin-SIRPα; five-folds higher in the aspect of cell binding ability, three folds higher of phagocytosic activity and 4 folds higher in the case of tumour growth inhibition.
Summary/conclusion: We compared the efficacy of two nanoparticles and concluded that exosome has more advantages in delivering membrane proteins for therapeutic purpose. Our findings highlight the ability of exosomes to display native membrane proteins on their surface – a significant advantage of this delivery system – and suggest that CD47 blockade by exosome-mediated SIRPα delivery is superior to that mediated by a protein scaffold.

LBS03.12

Cell-specific growth surface topography optimization for extracellular vesicle studies

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Introduction: While patient fluid samples provide valuable insight into the role of EVs in human health, their limited supply and heterogeneous nature make them impractical for basic studies. Conditioned media provides a consistent and limitless supply of EVs from a known cell type, but large volumes are required to generate adequate numbers of EVs. Also, little is known about how factors in the cellular microenvironment, like surface topography, affect the EVs due to a lack of accessible biomimetic cell culture systems. We present a unique cell culture dish covered in microtrack patterns and demonstrate that this biomimicry affects the EVs produced by cancer cells.
Methods: Microtrack patterns were fabricated using photolithography. Soft lithography was used to create microtrack moulds, which were spincoated with

polystyrene and stamped onto 150 mm petri dishes. Oxygen plasma and UV sterilisation were used to prepare the surfaces for cell growth. MCF7 breast cancer cells were seeded and cell viability and morphology were quantified. Live cells stained with Calcein-AM were imaged and their morphology was quantified using FIJI. Cytoskeletal structure was imaged using DAPI, TRITC-phalloidin and anti-vinculin/FITC-IgG. Cells were cultured in EV-depleted media for the last 48h and EVs from smooth (control) and patterned dishes were isolated using Vivaspin ultrafiltration and sequential ultracentrifugation. Finally, EV structural integrity, concentration and size distribution were characterized using TEM and nanoparticle tracking analysis.

Results: MCF7 cells cultured on microtrack dishes demonstrated similar viability to smooth surfaces. Cell morphologies on microtracks had higher average aspect ratios and less circularity ($p < .05$), as well as greater actin cytoskeletal alignment. Early nanoparticle tracking analysis results indicate that cells cultured on fibrous surfaces release more EVs than EVs from smooth surfaces and these results are currently being further corroborated.

Summary/conclusion: This type of patterned growth surface could have implications in both EV biomimicry and biomanufacturing. While it appears that simple surface patterning with microtracks could simply and inexpensively improve EV-yield from cell cultures, we are now exploring whether it also affects their biomimicry.

LBS03.13

Development of engineered extracellular vesicles expressing immune checkpoint protein PD-1 by fusion with liposomes

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Introduction: Extracellular vesicles (EVs) can potentially be used in biomedical applications in drug delivery system. However, many problems still remain, such as equipping EVs with active targeting ability and controlling the encapsulation of therapeutic drugs in EVs. We have developed a new method for the preparation of the functional EVs that display exogeneous membrane proteins using the baculovirus-expression system. In addition, we have proposed that EV – liposome hybrids were prepared by membrane fusion between liposomes and EVs containing fusogenic viral glycoprotein 64 (gp64). Here, we report preparation of new hybrid EVs expressing immune checkpoint protein PD-1 by this method and evaluation of the functions such as the specific interaction with cancer cells

Methods: The cDNA of PD-1 on a baculovirus vector was transfected into Sf9 insect cells, and EVs that were expressed PD-1 on the surface were collected by ultracentrifugation. The hybrid EVs were prepared by membrane fusion between PD-1 EVs and FITC-Dextran loaded-liposomes at the acidic condition. PD-1 and gp64 expression on PD-1 EVs and PD-1 hybrid EVs were detected by Western blotting. PD-1 hybrid EVs were incubated with Hela cells, and cellular uptake of PD-1 hybrid EVs was observed by confocal laser scanning microscopy (CLSM).

Results: As results of Western blotting, PD-1 and gp64 were detected on EVs and also hybrid EVs prepared at acidic pH. Membrane fusion between EVs containing gp64 and liposomes proceeded only under the acidic pH. Interaction between PD-1 hybrid EVs and PD-L1-expressing cancer cells was investigated by CLSM. The PD-1 hybrid EVs effectively internalized into the cells via interaction with PD-L1, and FITC-dextran (as a model of drug) loaded into PD-1 hybrid EVs was efficiently delivered into the cells.

Summary/conclusion: In summary, we prepared PD-1 hybrid EVs by using baculovirus-expression system and membrane fusion with functional liposomes. This method provides a new strategy for engineering EVs.

LBS03.14

Carcinogenesis and exosome packaging

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Introduction: Identification of cancer-specific biomarkers on exosomes has evaded researchers for years and poses an enormous roadblock towards developing effective diagnostic tools for early cancer detection. This is mainly due to the lack of a unique exosome marker. Therefore, if we intend to use exosomes as cancer biomarkers in liquid biopy we need to identify unique markers present on the exosomes or associated with them in cancer. Thus, we aim to delineate molecular mechanisms employed during exosome packaging and cargo-loading. We hypothesize that cancer cell employ distinct molecular machineries to package protein-cargo vs RNA-cargo into exosomes. We further postulate that understanding the packaging proteins involved in this cargo-specific packaging will reveal key stages of biogenesis and will aid in identifying

unique markers associated with exosomes during carcinogenesis.

Methods: Recombinant DNA technology, confocal, super-resolution imaging, ultracentrifugation, ultrafiltration and size exclusion columns, nanoparticle-tracking analysis electron microscopy, high-resolution flow cytometry and Western blotting

Results: We have generated an in-vitro cell culture system comprised of lung-cancer cell lines BEAS2-B and A549. The former represents early stage indolent disease while the latter represents late stage aggressive disease. The culture system is genetically engineered to stably express tagged components of an exosome, namely- lipid bilayer (Td-Tomato-tagged myristoylation sequence), transmembrane cargo protein (EGFP-tagged α5β6 integrin) and an RNA molecule (metabolically labelled). Exosomes generated by the system are isolated and characterized.

Summary/conclusion: Thus, we have generated a unique tool, with which we aim to identify – a) unique patterns of cargo packaging within these exosomes and b) to elucidate the proteins involved in this directed packaging. Research undertaken in this project will provide new and exciting avenues to detect signs of early cancer progression holds immense potential for identifying cancer biomarkers.

Funding: CEDAR (Cancer Early Detection Advanced Research Center), Knight Cancer Institute)

Symposium Session 26: Flow Cytometric Analysis of EVs
Chairs: Xiaomei Yan; Joshua Welsh
Location: Level 3, Hall B

16:00–17:00

OS26.01

Influence of lipoprotein particles in extracellular vesicle analysis by single particle flow cytometry

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Introduction: High-resolution flow cytometry (FC) allows for the detection of single extracellular vesicles (EV) and enables quantitative and qualitative characterization. EV in plasma has been associated with diseases, making them attractive for diagnosis and prognosis of patients. However, the presence of lipoprotein particles (LPP) in plasma may hamper robust flow cytometric analysis of EV. We here investigated the interference of these particles when generic fluorescent dyes are used for labelling and detection of EV by FC.

Methods: To define the impact of LPP on fluorescence-based FC–detection of EV, commercially available LPP preparations, EV isolated from conditioned media of the mouse 4T1 mammary carcinoma cell line, and platelet-poor plasma samples from healthy fastened human donors were stained with PKH67 and CFSE. EV was isolated from samples by differential ultracentrifugation or size-exclusion chromatography (SEC). Stained LPP, plasma EV and 4T1 EV were succumbed to density gradient floatation, after which FC-analysis was performed using a BD Influx that was optimized for detection of submicron-sized particles.

Results: We found that both PKH67 and CFSE have the capacity to label various types of LPP. When analysed by FC, fluorescently labelled LPP and EV are hard to discriminate based on fluorescent and light scatter signals. Interestingly however, both dyes show a different staining pattern for LPP and are indicative for the type of LPP analysed. In addition, we demonstrated that LPP show different sensitivity to detergent lysis when compared to EV. Finally, using spike-in experiments we found that the presence of LPP can obscure generic fluorescent labelling of EV, highlighting the need for proper EV isolation and purification when human plasma is used in generic fluorescent-based FC-detection of EV.

Summary/Conclusion: In order to perform reliable and reproducible fluorescent-based FC-analysis of single EV from human plasma, either EV-specific fluorescent dyes or labels should be used or plasma samples should be carefully cleared from particles prone to incorporate the generic dye.

Funding: European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No [722148] and STW-Perspectief Cancer-ID grant [14,191].

OS26.02

Single-particle analysis of exosome DNA/RNA abundance, identity and location via a laboratory-built nano-flow cytometer

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Introduction: Through packing and transferring nucleic acids including genomic DNA, mitochondrial DNA, microRNA, mRNA and long noncoding RNA, exosomes play important roles in maintaining cellular homeostasis, priming immune system and regulating tumour progression. However, the abundance, identity (single stranded or double stranded) and location (surface-bound or inside) of nucleic acids in single exosomes is still a conundrum. Herein, a laboratory-built nano-flow cytometer (nFCM) that enables multiparameter analysis of single exosomes as small as 40 nm is used to investigate the features of exosomal nucleic acids.

Methods: Exosomes derived from a colorectal cancer cell line (HCT15) and a normal colon fibroblast cell line (CCD-18Co) were isolated by differential ultracentrifugation. The location and identity of DNA/RNA was examined by measuring the fluorescence signals of single exosomes upon nucleic acid labelling with SYTO 16 or SYTO RNASelect dye before and after enzymatic digestion with DNase I, dsDNase, S1 nuclease and RNase A, respectively. To achieve a selective labelling for DNA/RNA, ethynyl-modified dUTP (EdU)/ethynyl-modified UTP (EU) were incorporated

into the newly synthesized DNA/RNA by metabolic biosynthetic pathway followed by chemoselective coupling with azide-AF488 via click chemistry.

Results: We found that the majority of exosomal DNA reside on the outer surface of exosomes via bound with membrane proteins and most of DNA are dsDNA. Meanwhile, almost all the RNA are encapsulated inside exosomes. Through correlation analysis with side scattering signals of single exosomes, it was identified that most of the luminal DNA are associated with large size exosomes and surface-adhering DNA mainly exist on small size exosomes; yet the particle size of RNA containing exosomes ranges from small to large sizes. Because exosomes maintain cellular homeostasis by excreting harmful cytoplasmic DNA from cells, the effect of anti-tumour agents such as etoposide, topotecan, SN-38 and cisplatin on the abundance and location change of exosomal DNA will be reported.

Summary/Conclusion: nFCM provides a straightforward and practical approach for the location and identity studies of nucleic acids in individual exosomes, which will be very helpful in the illustration of exosome-mediated, nucleic acids-based intercellular communication.

OS26.03

Using a combination of bead-based flow cytometry and imaging flow cytometry to understand Extracellular Vesicle heterogeneity

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Introduction: Extracellular vesicles (EVs) are secreted by all cell types and can be found in all body fluids. They can be roughly classified based on their size and origin as exosomes (70–150 nm) and microvesicles (100 nm to 1 µm). However, it is nowadays commonly accepted in the field that there is a much higher degree of EV heterogeneity within these two subgroups. Also, their content, protein composition and surface signature likely is dependent on multiple parameters like the cell's metabolic or immunological status. Moreover, the protein composition and surface marker signature of EVs is further dependent on the cell type releasing them. Accordingly, EVs secreted by different normal cell types or malignant cells also will display distinct surface profiles. Until today, only few EV surface markers have been related to specific cell sources.

Methods: We have recently optimized two flow cytometry based methods for EV surface marker analysis, a multiplex bead-based approach which allows robust identification of co-expressed surface marker combinations (Wiklander et al, 2018) and a method using imaging flow cytometry to quantify EV subsets at the single vesicle level (Görgens et al, in revision).

Results: Here, we combined both flow cytometric approaches aiming to identify EV surface marker combinations being specific for EVs from specific cell types and/or disease-related cells such as cancer cells. We first used the multiplex bead-based assay to screen EVs isolated from 40+ different immortalized human cell lines from different tissues. Next, we have applied the same screening technology to assess surface signatures of EVs derived from diverse biological fluids of human healthy donors in order to identify differential surface marker combinations between different body fluids and estimate general donor-to-donor variation within respective sample groups. Validation of identified EV surface signatures by high resolution single vesicle imaging flow cytometry and other methods is currently ongoing.

Summary/Conclusion: We will show preliminary data resulting from this approach and propose that the identification of specific EV surface marker combinations will be highly relevant to further understand the molecular content and related functions of subsets of EVs in health and disease.

OS26.04

A single extracellular vesicle (EV) flow cytometry approach to reveal EV heterogeneity

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Introduction: To reveal the clear correlation between extracellular vesicle (EV) functions and molecular signatures, the only effective approach is to analyse the molecular profile of individual EVs. Flow cytometry (FC) has been widely employed to distinguish different cell types in mixed populations, but the sizes of EVs fall well below the detection limit of conventional flow cytometers, making it impossible to do single-EV analysis without significant instrumentation development.

Methods: We innovatively solve this difficulty by amplifying the size of each EV by DNA nanostructures so that they can be analysed in conventional flow

cytometers. In this approach, either an aptamer or an antibody is employed to recognize the specific surface marker on each EV, and initiate construction of a large DNA nanostructure by hybridization chain reaction. The resultant structure not only enlarges the overall size of the single EV, but also can bind to multiple fluorophores to amplify the signal from the few number of molecules on the EV surface, enabling visualization of single EVs in a conventional flow cytometer.

Results: We have successfully demonstrated counting single EVs in the FACSCanto after a one-pot reaction, and multiple surface markers can be simultaneously targeted to differentiate EV sub-groups based on their surface protein signature. While aptamers provide a cleaner background for detection, the large selection of antibodies makes it applicable for diverse surface markers on the EVs for sub-grouping. We have been applying this technique to analyse EVs produced from different breast cancer cell lines, as well as the EVs in patients' sera.

Summary/Conclusion: In summary, we have developed a single-EV FC analysis technique to visualize single EV in a conventional flow cytometer. Our technique enables study of single EVs using this widely available instrument to gain in-depth insights into the molecular signatures of EV sub-populations at the single EV level. Targeting multiple markers greatly improves differentiation of EV sub-populations. The high simplicity of our method and its good adaptivity to clinical labs will be highly beneficial for screening for effective EV markers for liquid biopsy applications.

Funding: NIH-NCI

Symposium Session 27: Non-mammalian EVs
Chairs: Richard Ferrero; J. Max Silverman
Location: Level B1, Hall B

16:00–17:00

OS27.01

Extracellular vesicles released by commensal *Lactobacillus* suppress HIV-1 infection

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Introduction: The vaginal microbiota, mostly dominated by *Lactobacillus* spp. plays a key role in preventing from numerous uro-pathogens' infections, in particular from HIV-1. Recently, we demonstrated that *Lactobacillus* of various strains inhibit HIV-1 replication in human cervico-vaginal and tonsillar tissues *ex vivo* providing an experimental system to study mechanisms of this phenomenon. A growing body of evidences suggest that any kind of cells, including bacteria communicate to each other through extracellular vesicles (EVs). Here, we investigated whether the protective anti-HIV effect of lactobacilli is mediated by EVs released by these bacteria.
Methods: EVs were isolated from four strains of *Lactobacillus* cultures, previously isolated from vaginas of healthy women, by ultracentrifugation. Vesicles' sizes and concentrations were evaluated using NanoSight. Human cervico-vaginal and tonsillar tissues *ex vivo*, as well as cell lines were treated with *Lactobacillus*-derived EVs, infected with HIV-1 and virus replication was assessed by measuring the released capsidic protein p24 using Luminex. Protein and metabolite cargo of bacterial EVs were detected by LC/MS/MS and 1H-NMR analysis, respectively.
Results: EVs released by *L. crispatus* BC3 and *L. gasseri* BC12 protected human cervico-vaginal and tonsillar tissues *ex vivo* as well as isolated mammalian cells from HIV-1 infection by at least 50%. This protection was not due to cytostatic or cytotoxic EV-effects but rather was associated with the decrease of viral attachment to the target cell and viral entry as demonstrated in TZM-bl and MT-4 cell assays. Metabolomic analysis showed 42 molecules associated with EVs including amino acids, alcohols, ketones and monosaccharides. Proteomic analysis showed the presence of several bacterial proteins in EVs that may be associated with the anti-HIV effect.
Summary/Conclusion: Our findings demonstrate that the protective effect of *Lactobacillus* against HIV transmission is, in part, mediated by EVs released by these commensal bacteria. This finding may lead to new strategies to prevent male-to-female sexual HIV transmission.

OS27.02

Extracellular vesicles of the human gut microbiota: do you hear me host?

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Introduction: Microbial populations colonize the whole length of the human gastrointestinal track. Changes in composition and function of the gut microbiota have been linked with numerous pathologies, underlining the importance of the host-microbiota co-operation, although quite little is known of the mechanism of communication between microbiota and distal organs. Our aim was to describe EV secretion in healthy human gut, explore the contribution of different bacteria to EV secretion and characterize the cargo of gut microbiota EVs, our hypothesis being that EVs are one of the major communication systems between human gut microbiota and the host.
Methods: Gut microbiota EVs were isolated with a combination of commercial kits and centrifugation methods from 20 faecal samples from healthy donors. Presence of EVs was assessed with transmission electron microscopy (TEM). Proteins and RNA were isolated from the obtained vesicles and analysed with LC-ESI-MS/MS (Turku Proteomics Facility) and Illumina550 sequencing (Biocenter Oulu Sequencing

Centre). DNA was isolated from the faecal samples and analysed with 16S rRNA sequencing (Institute of Biotechnology, University of Helsinki) along with intact faeces-derived vesicles to allow comparison of taxonomic profiles.
Results: Populations of faecal EVs were detected with TEM, with a size ranging from 50 to 200 nm. On average, 184 bacterial proteins and 56 human proteins were identified per sample. Taken together, the data describes presence of 1194 distinct bacterial proteins and 264 human proteins in faecal EVs. On functional level, the majority of bacterial EV proteins of the gut seem to consist of outer membrane proteins relating to metabolism, bacterial invasion and transport. Data for RNA cargo analysis is pending. In terms of bacterial EV proteins, the data suggests the most diverse secretion from phyla bacteroidetes and firmicutes. Taxonomic profiles analysed by 16S rRNA sequencing demonstrated differences in the bacterial composition of the faecal samples and faeces-derived EVs: proteobacteria, while present in small abundancies in faeces, was one of the most predominant phyla found in faeces-derived EVs.
Summary/Conclusion: Human gut microbiota actively secretes EVs with range of protein and RNA cargo which biological significance in human health and disease needs to be studied further.
Funding: Academy of Finland

OS27.03

Preparation, characterization and cellular interaction of edible plant-derived nanoparticles

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Introduction: Nanoparticles, including liposomes, polymeric micelles and animal cell-derived extracellular vesicles (EVs), are promising carriers for bioactive molecules. Recently, edible plant-derived nanoparticles are expected to be a novel class of nanoparticles, because they have advantages in terms of mass production and cost-effectiveness. However, their pharmaceutical and biological characteristics need to be evaluated prior to their application and use in clinical practice. In this study, we selected corn as an edible plant, and prepared corn-derived nanoparticles (cNPs). Then, we evaluated their property and interaction with cells.
Methods: Corn was put in a blender with distilled water to obtain juice. The juice was separated by centrifugation and ultra-centrifugation (UC), and the pellet after UC at 100,000 g was collected as cNPs. The yield of the cNPs was evaluated by the protein amount measured using Bradford assay. The size and zeta potential of the cNPs were measured by a zeta sizer. To evaluate the effect of the cNPs on cells, three types of cell lines, i.e. murine fibroblast NIH3T3 cells, murine macrophage-like RAW264.7 cells, and murine colon adenocarcinoma colon26 cells, were selected. Cells were added with cNPs and incubated at 37°C for 24 h. The cell viability was evaluated by using CCK8 assay. Separately, the cNPs were labelled with DiI and labelled cNPs were added to cells. After incubation, we observed the cells by confocal microscopy.
Results: About 10 mg cNPs were obtained from 100 g plants, indicating that cNPs can be obtained with high yield compared with EVs. The size of the cNPs was about 200 nm. In addition, the zeta potential was a negative charge (about −15 mV), which is comparable to that of EVs. Low concentrations of cNPs hardly affected the viability of the cells. Confocal microscopy showed that DiI-labelled cNPs were taken up by RAW264.7 cells. The results of onion- or orange-derived NPs will also be presented.
Summary/Conclusion: We succeeded in preparing cNPs in large scale and revealed that the particulate properties of the cNPs are comparable to those of EVs. We also demonstrated that cNPs can be efficiently taken up by RAW264.7 cells. These results raise a possibility that cNPs can be used as carriers for bioactive molecules to such cells.

OS27.04

Biophysical and electrochemical characterization of redox-active extracellular vesicles from *Shewanella oneidensis*

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Introduction: Production of bacterial extracellular vesicles has been observed in marine and freshwater systems and in laboratory cultures. However, little is known about the function and mechanism of vesiculation in these nonpathogenic contexts. In addition to vesicles, the Gram-negative bacterium, *Shewanella oneidensis* also produces chains of outer-membrane vesicles that are proposed to function as bacterial nanowires for electron transport to solid-phase electron acceptors ranging from minerals to electrodes. A previous report demonstrated mineral reduction by isolated *S. oneidensis* vesicles. Many basic questions remain about the function and biogenesis of these

structures, particularly during metal and electrode respiration.

Methods: Here we report the purification and characterization of outer membrane vesicles from *S. oneidensis*. Preliminary analyses using dynamic light scattering, fluorescence microscopy, cryoelectron microscopy, and proteomics, confirm the size, content and reproducibility of purified vesicles.

Results: Proteomic data suggest that some proteins are selectively loaded into vesicles with the assistance of a novel BAR domain protein. Electrochemical analysis of surface deposited vesicles reveals the signatures of known outer-membrane multiheme cytochromes.

Summary/Conclusion: These results have implications for the role of vesicles and vesicle chains during respiration of iron oxides and anodes. Excitingly, this research suggest that a BAR domain protein provides the mechanistic relationship between vesicles and the outer membrane extensions known as nanowires

Funding: US DOE Division of Chemical Sciences, Geociences and Biosciences, Office of Basic Energy Science DE-FG02-13ER16415 National Science Foundation grant DEB-1542527

Symposium Session 28: EVs in Kidney and Urological Diseases
Chairs: Uta Erdbrügger; Juan Falcon-Perez
Location: Level B1, Hall A

16:00–17:00

OS28.01

Single MSC EV analysis for characterizing a subpopulation having therapeutic effects in AKI model

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Introduction: Therapeutic applications of MSCEVs have been extensively studied. Previous MSCEV studies demonstrated that MSCEVs showed various effects depending on how they were prepared. Recent studies suggested that this diversity might result from the heterogeneity of isolated EV populations. However, because of the absent of a proper EV subpopulation analysis method, no studies have succeeded to characterize an effective subpopulation from whole EV populations. We analysed the subpopulations of MSCEVs prepared by different isolation methods using a single EV analysis method. We assessed the correlation between the therapeutic effectiveness and MSC EV subpopulations using mouse acute kidney injury (AKI) model

Methods: EVs were prepared from hMSC conditioned media using different isolation methods: differential centrifugation, density gradient centrifugation and polymeric methods. A part of EVs were analysed using a TIRF microscopy based single EV analysis method, which can provide quantitative subpopulation information characterized by up to four different marker expressions. EVs were applied to an AKI model to assess their therapeutic effectiveness.

Results: EVs prepared by different isolation methods showed different subpopulation characteristics. The numbers of lipid marker positive EVs were different depending on their isolation method. Overall expression profile of three representative EV specific marker (CD9, 63 and 81) were also different depending on their isolation methods. EVs expressing more EV-specific markers were found to be more effective in mouse AKI models.

Summary/Conclusion: We demonstrated that the subpopulation composition of EVs prepared by different isolation methods were different. The numbers of EVs positive for multiple markers varied depending on the isolation methods. The relationship between therapeutic effectiveness and EV subpopulation marker expression were tested using an AKI model. EV subpopulation using four different EV-specific markers might be a useful tool for assessing the quality of isolated EVs in terms of their therapeutic effectiveness.

Funding: This work was supported by the KHIDI grant [HI16C2221] and supported by NRF grant [NRF-2018R1A2B3006280] funded by the Korean government.

OS28.02

Urinary microvesicular biomarkers for delayed graft function and overall outcome after living donor kidney transplantation

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Introduction: With a cargo of specific proteins and nucleic acids, urinary microvesicles represent a potential source for cellular material, that can be isolated easily and non-invasively. Yet, their clinical implementation in nephrology remains scarce with kidney biopsies still being the gold standard procedure in most diagnoses. We hypothesize that the addition of non-invasive biomarkers could benefit this invasive method with the potential risk of a sampling error.

Methods: With differential (ultra-)centrifugation, we isolated urinary microvesicles from living kidney transplant recipients and their donors over the course of 40 kidney transplantations. Whole urine samples were collected on day −1 (donor sample), 0, 1 and 3 months after transplantation (recipient sample). Microvesicular protein content was measured using quantitative mass spectrometry. We detected proteins, which linearly change their abundance in correspondence to clinical parameters, e.g. glomerular filtration rate (GFR) at 6 and 12 Months after transplantation in a set of 20 transplantations, by linear regression models. These

results were validated in a targeted proteomic screen in a cohort of 20 additional transplantations.

Results: We identified >1500 proteins present in at least 50% of the first sample set. Hierarchical clustering analysis depicted a clear clustering by time point of urine collection. Microvesicular proteins of glomerular (e.g. nephrin, podocin) or tubular origin (e.g. V-ATPase and Slc transporters) were regulated distinctly over the course of transplantation. Overall, specific proteomic time course patterns were apparent over the course of transplantation. Depending on low statistical error and high stability in a leave-one-out cross-validation of the linear models correlating to GFR values after transplantation, we created a list of 64 candidate proteins. Validation of these revealed PEPCK as a urinary microvesicular protein associated with GFR 12 months after transplantation.

Summary/Conclusion: With this study, we present the first analysis of the changes in the human urinary microvesicular proteome over the course of kidney transplantation. We believe, the validated biomarkers of all 40 Transplantations to hold the potential to further aid the diagnosis of graft survival.

Funding: MIWF Nachwuchsgruppen.NRW

OS28.03

Exosomal miRNA-19b-3p of tubular epithelial cell promotes M1 macrophage activation in kidney injury

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Introduction: Tubulointerstitial inflammation is a common characteristic for acute and chronic kidney injury. However, the mechanism by which the initial injury on tubular epithelial cells (TECs) drives interstitial inflammation remains unclear. Here we set out to characterize the miRNA profile of kidney exosomes and aim to explore the role of exosomal miRNAs derived from TECs in the development of tubulointerstitial inflammation.

Methods: Exosomes were isolated from kidney and characterized via electron microscopy and nanoparticle analysis. We examined expression profiles of miRNAs in kidney exosomes from LPS-induced kidney injury model by Exiqon microarray. Putative targets of miRNA were predicted by TargetScan. Chronic proteinuric kidney disease model was induced by adriamycin (ADR) administration. Exosomes purified from TECs were added to macrophages or intrarenal injected to mice to determine its effects both in vitro and in vivo.

Results: Global miRNA expression profiling on renal exosomes was examined in LPS-induced AKI model and miR-19b-3p was identified as the most remarkable miRNA increased in TEC-derived exosomes compared with controls. Similar results were found in ADR-induced chronic proteinuric kidney disease model in which exosomal miR-19b-3p was markedly released. Interestingly, once released, TEC-derived exosomal miR-19b-3p was internalized by macrophages, leading to M1 phenotype polarization through targeting NF-κB/SOCS-1. Importantly, the pathogenic role of exosomal miR-19b-3p in initiating renal inflammation was revealed by the ability of adoptive transfer of purified TEC-derived exosomes to cause tubulointerstitial inflammation in mice, which was reversed by inhibition of miR-19b-3p. Clinically, high levels of miR-19b-3p were found in urinary exosomes and correlated with the severity of tubulointerstitial inflammation in patients with diabetic nephropathy. Thus, our studies demonstrated exosomal miR-19b-3p mediated the communication between injured TECs and macrophages, leading to M1 macrophage activation.

Summary/Conclusion: Exosome/miR-19b-3p/SOCS1 axis played a critical pathologic role in tubulointerstitial inflammation that might represent a new therapeutic target for kidney disease.

OS28.04

A urine exosome RNA signature for prediction of high-grade prostate cancer: clinical validation in over 1,000 biopsy naïve patients

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Introduction: Discriminating indolent from clinically significant prostate cancer (PCa) prior to initial biopsy remains an important clinical and health economic issue. We have previously described the ExoDx Prostate(IntelliScore) (EPI) assay for discriminating high- vs low-grade prostate cancers using RNA extracted from urine exosomes. However proving efficacy and facilitating clinical adoption of a diagnostic assay requires extensive validation in prospectively collected patient cohorts. Here we compare performance of the EPI urine exosome assay vs. the Prostate Cancer Prevention Trial-Risk Calculator 2.0 (PCPT-RC) for

discriminating high-grade from low-grade PCa and benign disease on initial biopsy.

Methods: We collected data from two distinct validation cohorts (*N* = 519 and 503, respectively) representing 1022 subjects and compared EPI test results with biopsy outcomes. Eligible subjects were selected by age (>50-years) and PSA concentration (2–10 ng/mL), and were scheduled for initial prostate needle biopsy. Test performance was reported using the area under the receiver operating characteristic curve (AUC), negative and positive predictive value (NPV; PPV), sensitivity, and specificity. Outcome was based on Gleason Score (GS) for discriminating high- (GS≥7) from low-grade (GS = 6) and benign disease on initial biopsy.

Results: In this diverse cohort of 1022 biopsy naïve patients (mean age: 64 years, mean PSA: 5.6 ng/mL, ethnicity: 16% African, 71% Caucasian) we observed a 51% positive biopsy rate (30% GS≥7, 13% GS≥4 + 3). Performance of the EPI test (AUC = 0.70) was superior to PSA (AUC = 0.56), and PCPT-RC (AUC = 0.6; all *p*-values<0.001) for discriminating high- from low-grade PCa and benign disease. Using the previously validated cut-point of 15.6 (or alternative 20) would avoid 30% (or 43%) of unnecessary biopsies, with an NPV of 90% for both cut-points and miss only 7.5% (or 12%) of high-grade PCa patients.

Summary/Conclusion: EPI is a non-invasive 3-gene urine exosome RNA expression assay that we have now successfully validated in over 1000 patients to discriminate high- from low-grade PCa and benign disease. EPI identifies high-risk patients better than any current standard of care and provides a valuable tool for shared decision making so the right patients are sent for biopsy.

Symposium Session 29: Late Breaking- EV Therapeutics
Chairs: Masahiko Kuroda; Carolina Soekmadji
Location: Level B1, Lecture Room

08:30–09:30

LB01.01

First-in-human application of umbilical cord mesenchymal stromal cell-derived exosomes for the prevention of fibrosis following cochlear implant surgery

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Introduction: Cochlear implantation (CI) can restore hearing perception by bypassing the auditory hair cells (HC) and directly stimulating the spiral ganglion neurons (SGN). Insertion of an electrode array into the cochlea is associated with robust early and chronic inflammatory responses that promote intra-cochlear fibrosis and loss of HC and SGN. Conservation of residual hearing and prevention of fibrous tissue deposition around the electrode are thus major challenges in CI surgery.

Methods: We have manufactured GMP-compliant umbilical cord (UC)-MSC- derived extracellular vesicles/exosomes (EVs) and subjected such preparations to a series of *in vivo* and *in vitro* assays. Animal studies included both systemic and local injection for the improvement of seemingly unrelated indications such as critical size bone defects, partial tendon rupture and spinal cord injury in a rat contusion-model.

Results: In all cases EV application resulted in a significant modulation of immune reaction, overall reduced inflammation and scar reduction as evidenced by a reduction in ECM deposition. In an *in vitro* spiral ganglion neuron protection assay UC-MSC-EVs outperformed the current best-in-class soluble neuroprotective factor, BDNF. *In vivo* application of EVs in mice challenged by noise trauma resulted in significant protection of hearing when compared to untreated controls. Reduction of impedance as a measure for current resistance and fibrotic tissue formation around the electrode array were observed in a model of implantation trauma in guinea pigs implanted with an electrode array. After careful consideration of the medical history of a patient requiring CI surgery an experimental healing attempt was performed and the patient received a single intra-cochlear injection of 5 × 109 EVs (total) prior to electrode insertion. The application was tolerated well, no adverse reactions were recorded and

robust auditory sensation was detected 6 weeks post surgery.

Summary/conclusion: Based on these initial safety data on the local application of EVs in the inner ear, a phase 1/2a clinical trial is currently in preparation to further evaluate the neuroprotective, immunomodulatory and anti-fibrotic potential of UC-MSC-EVs.

Funding: "Exothera" IT-AT 1036 (EU), Project "ExtraNeu" (Land Salzburg)

LB01.03

Engineering of ARMMs for efficient delivery of Cas9 genome editors

Qiyu Wang[a] and Quan Lu[b]

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Introduction: Our previous studies have shown that the arrestin domain containing protein 1 (ARRDC1) drives the formation of extracellular vesicles known as ARMMs (ARRDC1-mediated microvesicles) (Nabhan J et al., PNAS 2012) and that these vesicles can be harnessed to package and deliver a variety of molecular cargos such as protein, RNA and the genome editor Cas9 (Wang Q and Lu Q, Nat Commun 2018). In the published packaging and delivery study, we used the full-length ARRDC1 protein (433 amino acids at ~46 kD) to recruit the molecular cargos into the vesicles, either through a direct fusion or via a protein-protein interaction module. Because ARRDC1 protein itself is packaged into ARMMs and because the size of the vesicles is limited (~80–100 nm), a smaller ARRDC1 protein that can still function in driving budding would potentially increase the number of cargos that can be packaged into the vesicles. Moreover, a smaller ARRDC1 may allow the recruitment of a relatively large cargo molecule.

Methods: We used protein engineering to identify a minimal ARRDC1 protein that can drive the formation of ARMMs. We then fused the minimal ARRDC1 to multiple proteins including the genome-editor Cas9 and tested the packaging and delivery efficiency of the fusion protein.

Results: Here we will present new data that identified a minimal ARRDC1 protein that contains an arrestin domain, PSAP and PPXY motifs. The minimal ARRDC1 is able to drive ARMM budding as efficiently as the full-length ARRDC1. We further present evidence that the minimal ARRDC1 protein can efficiently package cargos such as the relatively large Cas9/gRNA complex. In particular, we showed that the minimal ARRDC1 can package Cas9/gRNA into ARMMs through direction fusion whereas the full length ARRDC1 failed to.

Summary/conclusion: These results indicate that ARMMs formed by the minimal ARRDC1 may be used as a novel efficient therapeutic delivery platform for the Cas9-based gene editors.

LB01.04

Microvesicle-mediated delivery of minicircle DNA results in effective gene-directed enzyme prodrug cancer therapy

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Introduction: An emerging approach for cancer treatment employs the use of extracellular vesicles (EVs), specifically exosomes and microvesicles, as delivery vehicles.

Methods: We previously demonstrated that microvesicles can functionally deliver plasmid DNA to cells and showed that plasmid size and sequence determine, in part, the efficiency of delivery. Delivery vehicles comprised of microvesicles loaded with engineered minicircle DNA (MC) encoding prodrug converting enzymes were developed here as a cancer therapy in mammary carcinoma models.

Results: We demonstrated that MCs were loaded into shed microvesicles with greater efficiency than their parental plasmid counterparts and that microvesicle-mediated MC delivery led to significantly higher and more prolonged transgene expression in recipient cells than did microvesicles loaded with the parental plasmid. Microvesicles loaded with MCs encoding a thymidine kinase (TK)/nitroreductase (NTR) fusion protein produced TK-NTR expression in mammary

carcinoma cells. *In vivo* delivery of TK-NTR and administration of prodrugs led to the effective killing of both targeted cells and surrounding tumour cells via TK-NTR-mediated conversion of prodrugs to active cytotoxic agents. The efficiency of killing non-transfected bystander/neighbouring cells was assessed in mouse models and determined to require one in 100 cancer cells to be targeted.

Summary/conclusion: These results suggest that MC delivery via microvesicles can mediate gene transfer to an extent that enables effective prodrug conversion and tumour cell death such that it comprises a promising approach to cancer therapy. To understand the mechanism of this microvesicle-mediated enzyme prodrug therapy, we are currently assessing recipient cells in the tumour microenvironment.

Funding: This work was funded in part through a generous gift from the Chambers Family Foundation for Excellence in Pediatrics Research (to C.H.C.), Grant 1UH2TR000902-01 from the National Institutes of Health (to C.H.C.), and the Child Health Research Institute at Stanford University (to C.H.C.). Start-up fund from Michigan State University (to M.K.)

Symposium Session 30: Late Breaking- EVs and Cancer
Chairs: Suvendra Bhattacharyya; Vincent Hyenne
Location: Level B1, Hall B

08:30–09:30

LB02.01

Extremely-large extracellular vesicles (elevs) aid invasiveness of rasv12 tumour cell dissemination

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Introduction: Cancer cell dissemination has been recognized for the association with cancer recurrence, invasion and metastasis, however, the exact molecular mechanism is not fully understood. Most of the previous studies were conducted in cell culture, which is difficult to track the consequence of disseminated cells. Moreover, the lack of a simple yet conserved model system deferred genome-wide screening. Therefore, we established an *in vivo* cell dissemination model in *Drosophila*.
Methods: We express mutant Ras (RasV12) in adult Drosophila midgut intestinal stem cells (ISCs) and enteroblasts (EBs) using the conditional GAL4 driver esgts (esg-GAL4, tub-GAL80ts, UAS-GFP).
Results: When RasV12 is expressed in ISCs and EBs, tumour rapidly proliferates, then become eliminated. Cellular processes protrude while damaging and invading the surrounding visceral muscle fibres, and intact cells can completely disseminate. Interestingly, we observed with ex vivo live imaging that RasV12 cells produce large blebs and release extracellular vesicles. The average size of these vesicles was bigger than exosomes (<100 nm) and microvesicles (100–1000 nm), so we refer them as extremely-large extracellular vesicles (ELEVs). Additionally, GFP-positive particles were detected in haemolymph prepared from RasV12 flies but not from controls assuring that ELEVs were also produced *in vivo*. Of note, we found that metastatic RasV12, scrib-/- disc tumours also produced ELEVs. Thus, we propose that the generation of ELEVs is a characteristic of invasive tumours in Drosophila. Interestingly, these ELEVs are reminiscent of large oncosomes or cytoplasts, which have been implicated in the invasive behaviour of cancer cells.
Summary/conclusion: This model shares many known aspects of tumour cell dissemination implied from the studies in mammalian systems. We plan to utilize this unique system to elucidate the molecular mechanism for cell dissemination and ELEVs production using vast genetic tools available in Drosophila.

LB02.02

House dust extracellular vesicles promote tumour metastasis to the lungs by inducing tumour necrosis factor-α

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Introduction: Air pollution is associated with multiple pulmonary disorders. As a part of pollutant air, house dust harbours several biological contaminants including extracellular vesicles (EVs). House dust EVs have been shown to induce pulmonary inflammation, but no studies have assessed the effect of dust EVs on tumour metastasis to the lungs.
Methods: EVs were isolated from house dust using buoyant density gradient ultracentrifugation. Isolated dust EVs were characterized with transmission electron microscopy and dynamic light scattering. To assess the role of dust EVs in tumour metastasis, dust EVs were intranasally administered to mice, followed by intravenous injection of tumour cells after 1 day. At 2 weeks after tumour introduction, lungs were harvested from mice to measure metastasis by counting metastatic colonies. To investigate the mechanism, the lungs were collected at 12 h or 24 h after tumour cell introduction to access tumour cell infiltration into the lungs by immunohistochemistry. Furthermore, lung lysates were prepared from mice intranasally administered with dust EVs to examine tumour necrosis factor-α (TNF-α) production and their effect on tumour cell migration. Finally, TNF-α knock-out mice were used to show the importance of TNF-α in dust EV-induced tumour metastasis.
Results: House dust EVs had membrane-enclosed structures with an average diameter of 129.6 ± 4.5 nm, as observed by transmission electron

microscopy and dynamic light scattering. Dust EVs significantly promoted tumour metastasis to the lungs. The mechanism study showed that these EVs enhanced tumour cell infiltration into the lungs. Although dust EVs did not directly mediate tumour cell migration, lung lysates from dust EV-treated mice could promote this migratory effect. In addition, the concentration of TNF-α was increased in lung lysates by treating dust EVs. Finally, TNF-α knock-out mice treated with dust EVs could not promote tumour metastasis to the lungs.

Summary/conclusion: House dust harboured significant amounts of EVs which could promote tumour metastasis by inducing TNF-α. These findings provide mechanistic insights into the effect of house dust on tumour metastasis to the lungs.

LB02.03

Modeling tumour: key issues of cell communication by mean of EVs in a three-dimensional environment and the impact on biomarker discovery

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Introduction: The impact of growth architecture on intercellular communication, including EV release, cargo, and function was not examined in many details. In our recent work, we described a new model for efficient EV production in a three-dimensional environment. Cells growing in 3D produced an increased number of small EVs, which differed in their miRNA and protein profiles from the EVs, released by the cells grown under conventional conditions. In the current work, we describe the impact of growth architecture on EV heterogeneity and their content respecting recruitment of activated oncogenes and mutated products on EVs.

Methods: EVs of different size were isolated by subsequent sedimentation by 5000 ×*g* (EV5), 12000 ×*g* (EV12) and small EVs, purified by size exclusion chromatography (SEC). Subsequently, iodixanol gradient centrifugation was performed. These EV populations were separated from 2D and 3D cell cultures and characterized respecting their size using NTA, DLS, and TRPS. Surface proteins were examined using beads-assisted flow cytometry. Subsequently, DNA and RNA were isolated, and the number of mutated oncogenes to different EV populations was assessed using ddPCR.

Results: In all models, including prostate, breast, colorectal and gastric cancer, 3D environment caused a considerable shift in EV size distribution. Specific changes in EV surface protein profiles and distribution of oncogenic DNA and RNA among EV populations was observed. These changes varied between different tumour types. Comparison with the delivery of mutated oncogenes among EV populations in the blood of patients revealed that EVs released from in 3D resemble closer the content of EVs isolated from the blood of tumour patients.

Summary/conclusion: Here, we present a unique model applicable to study the EV heterogeneity under conditions mimicking physiological tumour microenvironment. It can serve as a new tool for drug screening and biomarker search, representing a new reliable model for EV-based liquid biopsy.

Funding: H2020 MSCA-ITN „Train-EV“, Project 722148; BMBF “EXONANSENS”, Project 01DJ51206.

LB02.04

Secretion mechanisms of wnt proteins

Alena Ivanova[a], Jan Winter[a], Oksana Voloshanenko[a], Kathrin Glaeser[a], Steffen Scholpp[b], Ulrike Engel[c], Clotilde Thery[d] and Michael Boutros[e]

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Introduction: Wnt signalling pathways play important roles in development and diseases of multicellular organisms. Key players in intercellular signalling – Wnt proteins – travel through extracellular space, but since lipid modifications renders them insoluble, they use special carriers. According to the current understanding of Wnt secretion, to contact neighbouring cells Wnt proteins can diffuse using heparan sulphate proteoglycan chains on the cell surface or they can be transported on filopodia. Additionally, Wnt ligands can be solubilized and travel in the intracellular space by creating lipoprotein particles. Wnts can be recycled through the endosomal compartment and secreted on exosomes. It is been a long debate which secretory mechanism is preferred by Wnt ligands and which forms of secreted Wnt proteins maintain signalling activity. We aimed to dissect the very complex Wnt secretion network to identify new regulators specific for the certain secretion roots.

Methods: We first, performed focused RNAi screen to identify new components of Wnt secretory pathway. By activating Wnt pathway either in the secreting cell or at the receptor level we manage to distinguish between proteins that involved only in the secretory part of the pathway or play role in the receiving cells. At the next step, we used array CRISPR/Cas9 screening approach for targeted disruption of genes together with canonical Wnt activity assay to confirm that identified genes are required for the secretion of functional Wnt proteins.
Results: With the described approach, a panel of 83 possible secretory factors have been tested. Among the others we found a protein that involved in the filopodia formation process. Identified protein regulates number and length of cell filopodia. Beyond this, only a few other proteins have been described so far to regulate specialized filopodia like cytonemes. Additionally, we observed that Wnt proteins travel across filopodia being packed on vesicle-like structures.
Summary/conclusion: For the first time a forward genetic screen allowed to identify new components that are important for filopodia associated Wnt signalling. Surprisingly, Wnt ligands use vesicles as carries for transport across cell protrusions. These findings add another piece of evidence that microvesicles and filopodia plays a significant role in the distribution of Wnt ligands.

Symposium Session 31: Late Breaking- EV Biomarkers
Chairs: Johannes Grillari; Mariko Ikuo
Location: Level B1, Hall A

08:30–09:30

LB03.02

Assessing the value of extracellular vesicles' DNA and proteins as biomarkers in metastatic breast cancer

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Introduction: Analysis of cell-free circulating tumour DNA (ctDNA) and cancer-specific extracellular vesicles (EVs) in patients' blood have been widely explored as biomarkers for cancer detection and disease follow up. These non-invasive biomarkers represent a promising tool for real-time monitoring of treatment efficacy. Particularly, tumour-derived EVs contain specific protein cargo and nucleic acids, which are protected from degradation. However, most of the protocols used to isolate EVs co-isolate other nucleic acids carriers and the actual value of EV-associated nucleic acids as robust biomarkers remain unclear. Here, we assessed the clinical validity of nucleic acids specifically derived from EV-enriched fractions in comparison to non-EV fractions and total plasma as a source of specific and sensitive biomarkers in breast cancer.

Methods: Healthy donors or metastatic breast cancer patient's plasma (collected under patient written consent) was subjected to size exclusion chromatography to separate EVs (EV fraction) from other circulating components (soluble fraction). We quantified different DNA species present in these fractions as compared to total plasma. Nuclear and mitochondrial DNA (gDNA and mtDNA) were quantified by qPCR. Tumour specific nuclear alleles were detected by droplet digital PCR targeting known point mutations (previously identified from the tumour of each patient). Finally, 37 EV proteins were analysed using the MACSPlex Exosome Kit (Miltenyi).

Results: gDNA and mtDNA were both detected in EV fractions. However, gDNA content (total or mutant alleles) detected in the EV fractions was lower than in the soluble fractions and total plasma. In contrast, mtDNA was preferentially enriched in EV fractions. We observed similar levels of mtDNA or gDNA in cancer patients and healthy donors in the EV fractions, whereas in total plasma, gDNA was increased in cancer patients. In addition, several membrane proteins were significantly enriched or exclusively present in cancer patients EVs compared to healthy donors.

Summary/conclusion: Our findings provide evidence that the detection of DNA within total circulating EV does not provide added value as compared to the whole plasma. However, analysis of a subtype of EV-associated proteins may reliably identify cancer patients.

Funding: INCa-11548, ARC PGA1 RF20180206962

LB03.03

A novel strategy for early detection of clinically significant prostate cancer by high-throughput palmitoyl-proteomics of extracellular vesicles

Dolores Di Vizio[a], Javier Mariscal[b], Tatyana Vagner[b], Minyung Kim[b], Bo Zhou[c], Desmond PINK[d], Andrew Chin[b], Mandana Zandian[b], John Lewis[e], Michael Freeman[b], Stephen Freedland[b], Sungyong You[b], Wei Yang[b] and Andries Zijlstra[f]

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Introduction: Early diagnosis of lethal prostate cancer (PC) is critical for treatment stratification. Extracellular Vesicles (EVs) are an appealing source of circulating biomarkers. We sought to perform a state-of-the-art palmitoyl proteome to identify markers of aggressive PC because we noticed an enrichment for putative palmitoylated proteins in EVs in comparison with cells, and because most of the plasma proteins that contaminate the EV preps are not palmitoylated. Palmitoylation is a post-translational modification that anchors proteins transiently to the membrane. We reasoned that this could be a mechanism to anchor proteins temporary to the membrane and shed them in EVs.

Methods: Discontinuous centrifugation gradient, tunable resistive pulse sensing (QNano), next-generation PalmPISC for highly selective enrichment of palmitoyl-proteins, 2D LC-MS/MS for deep proteomics profiling, Nano-Flow Cytometry (Apogee), Western blotting.

Results: We isolated large and small EVs from PC3 cells and confirmed their biochemical and biophysical identity. We observed enrichment of distinct palmitoyl-proteins in both populations of EVs versus the

cells of origin. Pathway analysis demonstrated a strong association between large EV cargo and protein localization and small EV cargo and metabolic activity. Interestingly, palmitoyl-CD63 was enriched in large EVs while the total protein is enriched in small EVs. Similarly, palmitoyl-HSPA5 was enriched in small EVs, while the total protein is enriched in large EVs. This result suggests that the palmitoyl proteome might reveal a pool of markers that would not be identified otherwise. The Six Transmembrane Epithelial Antigen Prostate 1 (STEAP1) was enriched in EVs from aggressive cancer cells but not in the cell themselves, suggesting that it might be shed and thus identified in plasma of patients with aggressive disease even if it is not enriched in the tumour tissue. We interrogated a cohort of benign (n = 30), low Gleason Score (GS) (n = 30) and high GS (n = 30) patients. The number of samples with detectable STEAP1 expression was negligible in men with benign disease, and a significantly more frequent event in patients with high vs low GS.

Summary/conclusion: This study suggests that identification of bonafide palmitoylated proteins in EVs represents a viable liquid biopsy to identify lethal prostate cancer.

LB03.04

Circulating exosomal PD-L1 as a marker for the follow up of melanoma patients

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Introduction: In the era of effective molecular targeted treatments and immunotherapies, there is an urgent need to implement the use of circulating biomarkers in the clinic to facilitate personalized therapy and predict treatment response. We conducted a prospective study to demonstrate the involvement of circulating PD-L1 exosomes in melanoma patients.

Methods: One hundred melanoma patients were included. Exosomes were isolated by ultracentrifugation and evaluated by nanoparticle tracking analysis using a NTA technology. Isolated exosomes were tested for the expression of exosomal markers such as TSG101. PD-L1 expression in plasma and in melanoma plasma-derived exosomes (ExoPD-L1) was measured using an enzyme-linked immunosorbent assay.

Results: First, ExoPD-L1 was assessed in melanoma cell lines. ExoPD-L1 have a role in cancer immunosuppression mediated by T-cells since they were as efficient as cancer cells to inhibit T-cells activation. In melanoma patients, ExoPD-L1 (median 64,26 pg/mL) was significantly higher than free PD-L1 in the plasma which was barely detectable (0,1 pg/mL). Furthermore, ExoPD-L1 was detected in all patients whereas only 67% of the tumours were positive for PD-L1. Although baseline ExoPD-L1 levels were not associated with clinicopathologic characteristics and tumour burden, ExoPD-L1 variations (ΔExoPD-L1) after treatment correlated with tumour response and survival. A ΔExoPD-L1 cut-off of > 100 was defined, yielding a 83% sensitivity, a 70% specificity, a 91% positive predictive value and a 54% negative predictive values for disease progression. The use of this cut-off allowed stratification in two groups of patients statistically different in terms of overall survival and progression free survival.

Summary/conclusion: PD-L1 level in circulating exosomes may be a more reliable marker than PD-L1 expression in tumour tissue. Circulating exosomal PD-L1 monitoring may be a promising biomarker to predict tumour response and the clinical outcome.

Symposium Session 32: Late Breaking- EV Labeling, Separation, and Detection
Chairs: Elisa Lazaro-Ibanez; Ryou-u Takahashi
Location: Level B1, Lecture Room

09:30–10:15

LB04.01

A microfluidic device with nanoscale surface topology and functionalized with lipid nanoprobes for extracellular vesicle isolation and clinical cancer diagnosis

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Introduction: Extracellular vesicles (EVs) are cell-derived, lipid membrane enclosed particles. Tumour cell-derived are increasingly recognized for their pathophysiological contributions and potential towards cancer diagnosis and treatment monitoring. However, clinical translation of EVs has been limited by technological challenges for EV isolation. A rapid, high-throughput, and on-chip EV isolation technology is critical for EV-based cancer diagnosis.

Methods: We report a lipid nanoprobe-functionalized nanostructured silica microfluidic device that can be used in combination with nucleic acid extraction, and digital droplet polymerase chain reaction (ddPCR) for EV isolation, enrichment, and DNA mutation detection from clinical plasma samples for cancer diagnosis. The device consists of EV-size-matched silica nanostructures, surface-grafted lipid nanoprobes and a polydimethylsiloxane (PDMS) herringbone micromixer chamber. Plasma samples are collected from either cell lines or clinical samples (IRB approved and patients consented). As plasma flows through the microfluidic device, the EVs are isolated. EV DNA is then extracted and pathological mutations are detected with ddPCR.

Results: The microfluidic device removes 96.5% plasma proteins. The limit of detection of a KRAS mutation from plasma EV by ddPCR is 0.01% mutant allele fraction (MAF). The device is validated in a pilot clinical study for pancreatic cancer diagnosis. Clinical samples with known KRAS mutations in the tissue were validated with the device. ddPCR indicated MAF of 1.8%, 10.1%, and 22.3%, respectively, from DNA extracted from plasma EV, while none were detected in healthy controls.

Summary/conclusion: This new platform suggests that MAF of EV-derived DNA can have large patient variability that may depend on cancer type, stage, progression, or other pathophysiological factors. These results support the need for a rapid and reliable EV isolation technique, such as this reported device.

Funding: This work was supported by the National Cancer Institute of the US National Institutes of Health under grant number 1R01CA230339 to S. Y. Zheng.

LB04.02

Asparagine-linked glycosylation amplifies the heterogeneity of tumour extracellular vesicles

Yoichiro Harada[a], Kazuki Nakajima[b], Nobuyoshi Kosaka[c], Tomoko Fukushige[d], Kiyotaka Kondo[a], Junichi Seino[e], Tadashi Suzuki[e], Hiromasa Inoue[a], Takuro Kanekura[f], Takahiro Ochiya[c] and Ikuro Maruyama[a]

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Introduction: Tumour cells secrete heterogeneous populations of extracellular vesicles (EVs) carrying distinct proteins. However, the molecular underpinnings that regulate such EV heterogeneity remain largely elusive. Tumours consume a large quantity of glucose through glycolysis for the synthesis of various bioactive metabolites.

Methods: EVs were prepared from conditioned medium of mouse B16-F10 melanoma cells by differential centrifugation. The number of EVs secreted, their cargo proteins and intracellular carbohydrate metabolism were analysed.

Results: Here, we show that 2-DG, a glycolysis inhibitor, suppressed secretion of melanoma EVs independently of its glycolysis blockade action. 2-DG-sensitive EVs were enriched with asparagine (N)-linked glycosylated proteins, while 2-DG-resistant EVs contained intrinsically non-glycosylated proteins. Metabolic conversion of 2-DG to artificial nucleotide sugars via glycolysis branches induced degradation of N-linked glycan precursors and hypoglycosylation of multiple glycoproteins. Mutagenesis at N-linked glycosylation

sites of an EV cargo protein or pharmacological inhibition of N-glycosylation reaction by oligosaccharyltransferase was sufficient to suppress secretion of N-linked glycosylated proteins by EVs.
Summary/conclusion: This study establishes N-linked glycosylation as a key posttranslational modification that influences the heterogeneity of tumour-derived EVs.

LB04.03

Characterization of fluorescent plasma evs following 5-ALA use in malignant gliomas.

Leonora Balaj[a], Pamela Jones[b], Anudeep Yekula[b] and Bob Carter[b]

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Introduction: Malignant gliomas are rapidly progressive brain tumours with very high morbidity and mortality. The recent FDA approval of 5-aminolevulinic acid (5-ALA, *Gliolan*) provides the neurosurgeon with real-time fluorescent delineation of malignant tissue which allows a significantly higher rate of complete resections of malignant gliomas and longer progression-free survival compared to conventional white-light resections. We sought to determine whether fluorescent EVs would be released in the plasma of these patients.
Methods: Here, we characterize EVs isolated from glioma cell lines treated with 5-ALA for 24 h. We also evaluated plasma-derived EVs from glioma patients following preoperative oral administration of 5-ALA. We used a highly sensitive fluorescence-based analysis known as Amnis ISX mkII imaging flow cytometer to measure fluorescent signals from individual nanoparticles with the added value of being able to individually visualize particles being measured.
Results: We first compared the rate of EVs released from glioma cells treated with 5-ALA and determined a significant number of fluorescent EVs released within hours of exposure to 5-ALA, while the healthy human brain microvascular endothelial cells (HBMVEC) did not release any fluorescent EVs. We also compared the direct analysis of conditioned media to that of EVs purified by a commercial kit and determined that the extra exposure to light of EVs with the commercial kit leads to a significant loss of fluorescent EVs. To confirm our findings we exposed 5-ALA EVs to white light for 20 min and compared the number of fluorescent events before and after exposure to light, and determined a > 98% loss of fluorescent EVs. Finally, a comparison of the plasma samples from glioma patients collected upon administration of 5-ALA revealed that we can reliably detect fluorescent EVs in the plasma of these patients when the primary tumour fluoresces, while these events were undetectable in the cases where the primary tumour did not fluoresce. Furthermore, these events were undetectable upon tumour resection.
Summary/conclusion: This study is as a proof of concept to determine our ability to utilize fluorescent based tumour-specific EV characterization to aid in the diagnostics and prognostics of gliomas.
Funding: CA069246 CA230697 TR000931

Symposium Session 33: Late Breaking- From Biogenesis to Uptake
Chairs: Yutaka Naito; Ganesh Shelke
Location: Level B1, Hall B

09:30–10:15

LB05.01

Reassessment of exosome composition

Dennis Jeppesen[a], Aidan Fenix[b], Jeffrey Franklin[a], James Higginbotham[a], Qin Zhang[a], Leonard Rome[c], Dylan Burnette[b] and Robert Coffey[a]

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Introduction: The heterogeneity of extracellular vesicles (EVs) and presence of non-vesicular extracellular nanoparticles pose major obstacles to our understanding of the composition and functional properties of distinct secreted components. Greater precision in assigning RNA, DNA and protein to their correct extracellular compartments and identifying their mechanisms of secretion is crucial for identification of biomarkers and design of future drug interventions.

Methods: We have employed high-resolution density gradient fractionation and direct immunoaffinity capture (DIC) to precisely characterize the RNA, DNA, and protein constituents of exosomes and other non-vesicle material. Proteomics and RNA-Seq analyses were performed on purified small EVs and extracellular non-vesicular material. DIC was used to specifically isolate exosomes from other types of small EVs and was performed without ultracentrifugation and with capture beads targeting the classical exosomal tetraspanins CD63, CD81 and CD9. Biochemical analysis and structured illumination microscopy were used to examine secretion and presence of extracellular DNA.

Results: Extracellular RNA, RNA-binding proteins and other cellular proteins are differentially expressed in exosomes and non-vesicle compartments. Argonaute 1–4, glycolytic enzymes and cytoskeletal proteins were not detected in exosomes. We further demonstrate that small EVs are not vehicles of active DNA release. Instead, we propose a new model for active secretion of extracellular DNA through an autophagy- and multivesicular endosome-dependent but exosome-independent mechanism.

Summary/conclusion: This study demonstrates the need for a reassessment of exosome composition and offers a framework for a clearer understanding of EV and extracellular nanoparticle heterogeneity.

Funding: This study was part of the NIH Extracellular RNA Communication Consortium paper package and was supported by the NIH Common Fund's exRNA Communication Program. The work was funded by NIH grants The work was funded by NIH grants F31 HL136081 to Aidan M. Fenix, R35 GM125028 to Dylan T. Burnette, and R35 CA197570 and U19 CA179514 to Robert J. Coffey

LB05.02

Biofunctional peptide-modified extracellular vesicles for targeted intracellular delivery

Ikuhiko Nakase

Graduate School of Science, Osaka Prefecture University, Sakai-Shi, Japan

Introduction: Our research group is developing therapeutic techniques based on extracellular vesicles (exosomes, EVs) and peptide chemistry to deliver therapeutic/diagnostic molecules into targeted cells. Because of pharmaceutical advantages of the EVs as carriers for intracellular delivery of therapeutic molecules, we are trying to develop methodology to easily modify biofunctional peptides on exosomal membranes for receptor target and enhanced cellular uptake of the EVs. In this presentation, modification techniques using biofunctional peptides such as arginine-rich cell-penetrating peptides (CPPs, macropinocytosis induction) [1], artificial coiled-coil peptides (receptor target) [2], membrane fusion peptides (cytosolic release) will be introduced [3, 4]. And newly developed exosomes decorated with cell-penetrating sC18 peptides [5], which are derived from cationic antimicrobial protein, CAP18, will be also presented and discussed for cancer targeting.

Methods: For cellular uptake assessments of EVs, we used CD63 (EV marker protein)-GFP-fusion protein expressed EVs. All biofunctional peptides were synthesized by Fmoc solid-phase methods.

Results: Macropinocytosis has been shown to be very important for cellular EV uptake [1]. Therefore, our research group developed the methods for modification of arginine-rich CPPs on EV membranes using chemical linkers or acylation technique, which can induce clustering of proteoglycans (e.g. syndecan-4) and macropinocytosis signal transduction [1]. In the

research of artificial coiled-coil peptides, the artificial leucine zipper peptide-modified EVs recognize the peptide-tagged receptor expression on targeted cells [2]. Stearylation of branched sC18 peptides were easily modified on the EVs by their insertion of hydrophobic moiety in EV membranes, resulted in effective induction of macropinocytosis and cancer cellular uptake.

Summary/conclusion: These experimental techniques will contribute to development for the EV-based targeted intracellular delivery systems.

Reference: [1] I. Nakase, et al. Sci. Rep. 6, 34937 (2016), [2] I. Nakase, et al. Chem. Commun. 53, 317 (2017), [3] I. Nakase, et al. Sci. Rep. 5, 10112 (2015), [4] M. Akishiba, et al. Nat. Chem. 9, 751 (2017), [5] A. Gronewold, et al. ChrmMedChem. 12, 42 (2017)

LB05.03

Virus protein pX facilitates naked particles of hepatitis A virus to acquire an exosome-derived membrane by interacting with ESCRT-associated protein ALIX

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Introduction: Hepatitis A virus (HAV), a classically-thought non-enveloped virus, has recently been found to release majorly in the form of quasi-enveloped HAV (eHAV) by hijacking the host’s endosomal sorting complexes required for transport (ESCRT) complexes. Compared to the non-enveloped virion, eHAV exclusively contains a viral protein pX.

Methods: Differential centrifugation and iodixanol-based gradient centrifugation were used to isolate different types of EVs. Western-blot, Nanoparticle tracking analysis, and immune-electron microscopy were used to analyse EVs and HAV virus particles. Fluorescence microscopy in live-cell and immune-electron microscopy was used to identify the exosome-like biogenesis of eGFP-pX. Co-IP was performed in 293T cells. Amino-acids truncation and mutation in pX were performed in order to find the novel functional domain of pX.

Results: Fusing pX to eGFP could guide eGFP into exosomes through directing eGFP into multivesicular bodies (MVBs). Simultaneously, the release of ALIX, an MVBs maker protein, is enhanced and co-IP assay confirms the direct interaction between pX and V domain of ALIX. Furthermore, deleting C-terminal half of pX abolishes eHAV release and the interaction between HAV virion and ALIX. Consistently, the C-terminal half of pX alone is sufficient to load eGFP into exosomes by interacting with ALIX. On the other side, we find there is a novel nucleus export signal (NES) motif MMSRIAAGD in its N-terminal 12 amino-acids, which was previously reported essential for virion assembly

Summary/conclusion: Here, we find that pX is required for eHAV release and contains a novel nucleus export signal (NES) in its N-terminal. To conclude, pX plays important roles in HAV assembly and release through two independent domains: the N-terminal domain containing a novel NES, and the C-terminal half domain associated with the membrane envelopment via the interaction with ALIX. Thus, pX may have the potential for protein loading into exosomes in the future.

Funding: This work was supported by the national natural Science Foundation of China (81772200).

Symposium Session 34: Late Breaking- EV Signatures and Function
Chairs: Ter-Ovanesyan; Yusuke Yoshioka
Location: Level B1, Hall A

09:30–10:15

LB06.01

Proteomic and miRNA transcriptome analysis revealed an association between circulating exosomal miRNAs and insulin sensitivity in gestational diabetes mellitus during gestation

Soumyalekshmi Nair[a], Dominic Guanzon[b], Andrew Lai[c], Katherin Scholz-Romero[c], Carlos Palma[c], Jezid Miranda[d], Fatima Crispi[e], David McIntyre[f], Martha Lappas[g] and Carlos Salomon[b]

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Introduction: Gestational Diabetes Mellitus (GDM) is the most common medical complication in pregnancy, with short and long term metabolic effects in mothers and offsprings. We comprehensively analysed the exosomal miRNA profile across gestation in normal glucose tolerant (NGT) and women with GDM and determined the signalling pathways associated with changes in miRNA profile.
Methods: Exosomes were isolated from plasma samples collected at three time points during pregnancy from NGT and GDM women. Using a small RNA library and linear mixed modelling analysis, the miRNA profiles across gestation in NGT, GDM and NGT vs GDM were identified in a discovery cohort and the expression of candidate miRNAs were measured using qRT-PCR in a validation cohort. Further, we characterized the changes in the proteomic profile in skeletal muscles obtained from GDM patients compared to NGT controls, using a quantitative, data-independent acquisition mass spectrometric approach and finally integrated the exosomal miRNA and skeletal muscle protein expression profiles to identify miRNA-targeted networks.
Results: A total of 279 (NGT), 308 (GDM) and 175 (NGT vs GDM) miRNAs were significantly changing in expression across gestation. 6 miRNAs (hsa-miR-92a-3p, hsa-miR-10a-5p, hsa-miR-151b, hsa-miR-16–2-3p, hsa-miR-1910-5p and hsa-miR-423-5p) were confirmed to be differentially expressed in GDM. Proteomic characterization revealed 55 proteins to be differentially expressed in GDM skeletal muscles compared to NGT. The exosomal miRNAs upregulated in GDM target some of these differentially expressed proteins (Serine/Threonine Protein Phosphatase 6 (PPP6), Chloride Intracellular Channel Protein 4 (CLIC4) and Actin Related Protein Complex 2 (ARPC2)) in skeletal muscles in GDM and associated with pathways regulating glucose metabolism and insulin signalling (such as STAT 3 pathway).
Summary/conclusion: The miRNA content in maternal circulating exosomes differs across gestation in GDM patients compared to NGT and target specific proteins and pathways in skeletal muscle. This suggests that exosomes may be involved in maternal metabolic adaptation to pregnancy through the delivery of bioactive miRNAs.
Funding: Diabetes Australia, Lions Medical Research Foundation, NHMRC; 1114013, and FONDECYT 1170809.

LB06.02

Extracellular vesicles from induced neurons trigger epigenetic silencing of a brain neurotransmitter

Glenn McConkey[a], Isra Alsaady[b], Ellie Tedford[c] and Norhidayah Badya[d]

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Introduction: Our new breakthrough finding is that extracellular vesicles (EVs) injected into the brain specifically down-regulated production of the neurotransmitter norepinephrine suppressing transcription of the DBH gene and hypermethylation of the gene's promoter. DBH produces norepinephrine from dopamine in neurons. Previous studies found EVs regulate immune responses via PTGS but regulating neurons and

epigenetic changes have not been described. DNA methylation in neurons is involved in memory and neurological disorders (*Science* 2018 361 (6409)). These observations concur with our recent study that found central noradrenergic signalling is suppressed in the brains of infected rodents and in neurons (*Infect Immun* 2019 87(2)) for this parasite that causes movement disorders and is associated with neurological disorders.

Methods: Neuronal cells were induced by infection with the neurotropic protozoan Toxoplasma gondii and EVs purified on sucrose gradients. EVs, characterized by TEM, were used to treat rat and human neuronal cells and DBH mRNA and nascent DBH gene transcription were measured. DNA methylation was measured by MSRE-qPCR. Induced EVs were injected into the locus co-eruleus of rats and DBH gene expression was monitored.

Results: We found that EVs purified from infected neuronal cultures (43 ± 5 nm) specifically caused transcriptional gene silencing (TGS) and DNA methylation in noradrenergic neurons. The induced EVs down-regulated DBH gene expression >200-fold and, surprisingly, the down-regulation was at transcriptional level. The EVs also caused an epigenetic change; specifically inducing DNA hypermethylation of the DBH gene. Intracerebral injection of induced EVs into rats down-regulated DBH expression. We are currently identifying the RNA responsible as the down-regulation was disabled by degradation of the small RNAs in the EVs.

Summary/conclusion: This is the first study to find transcriptional gene silencing of a neurotransmitter in the brain by EVs and DNA hypermethylation in the neurons. This research will enhance our understanding of neurological disorders (ie. schizophrenia, epilepsy, drug addiction) and how memory works. The role of EVs in regulating neurotransmission in the brain will be presented.

LB06.03

Extracellular vesicles from human iPS-derived cardiovascular progenitors do not trigger an immune response in the infarcted heart

Bruna Lima Correa[a], Nadia EL-Harane[b], Ingrid Gomez[b], Hocine Rachid[c], José Vilar[d], Manon Desgres[e], Valérie Bellamy[e], Laetitia Pidial[e], Paul Alayrac[e], Dominique Charron[f], Nisa Renault[g], Reem Al-Daccak[h], Jean-Sebastien Silvestre[e] and Philippe Menasché[i]

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Introduction: Extracellular vesicles (EV) recapitulate most of the cardioprotective effects of stem cells but their immunological impact remains poorly understood.

Hypothesis: Immune response to EV may be beneficial rather than deleterious for the infarcted heart.

Methods: EV secreted from human-induced pluripotent stem cells [EV-hPg-iPS] were first assessed *in vitro* for the expression of immune and stem cell markers by flow cytometry and their cross-talk with allogeneic T and NK cells, was determined by mixed lymphocyte reactions (MLR). Then, 70 immunocompetent mice underwent a myocardial infarction and surviving mice were injected intramyocardially (under echo guidance) with EV-hPg-iPS, hPg-iPS or PBS either acutely ($n = 6$) or chronically ($n = 6$), i.e., 3 days and 3 weeks after infarction, respectively. Immune responses were monitored 3 days after treatment in all mice. Eighteen additional animals were sham-operated and also injected after 3 weeks with EV-hPg-iPS, hPg-iPS or PBS. Pro- and anti-inflammatory cytokines were measured in heart tissue and plasma by a bead-based multiplex immunoassay ($n = 6$/group).

Results: EV-hPg-iPS expressed stem cell markers (SSEA-1, CD15, CD133) and low levels of HLA class I and PD-L1. MLR and *in vivo* studies demonstrated that EV do not activate an adaptive allogeneic immune response since they failed to induce proliferation of allogeneic CD8+ or CD4 + T cells. In contrast to their parental cells, EV did not induce NK cell degranulation either. While injection of hPg-iPS or their EV at the chronic post infarction stage did not affect the number of T cells, B cells, and macrophages in cardiac tissue, spleen, bone marrow and blood, they significantly decreased the circulating levels of the pro-inflammatory cytokines IL-1α and IFN-γ compared to the control group (PBS). However, at the acute stage, and in contrast to PBS, EV significantly reduced the number of monocytes High (M1 macrophage precursor), M1 macrophages, and neutrophils as well as the circulating levels of the pro-inflammatory cytokines IL-1α, IL-2 and IL-8 while it significantly increased those of IL-10.

Summary/conclusion: EV-hPg-iPS look immunologically neutral *in vitro* and *in vivo* and seem even able to mitigate the infarct-related inflammatory response.

Funding: INSERM, LabexRevive, APHP, University Paris Descartes, Fondation de France, FRM